Experimental DNA Vaccine against Filariasis

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Declaration

I declare that the contents of this thesis are completely my own work, unless stated otherwise.

Honglin Luo

To my parents Kai and Rong,

Wife Joan and

Son Jonathan

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Abstract

Filarial infections are major causes of morbidity in the tropics and sub-tropics, afflicting over 150 million people in about 80 countries, causing debilitating symptoms such as elephantiasis (lymphatic filariasis), dermatitis and blindness (onchocerciasis or river blindness). Current control of lymphatic filariais relies on mass drug treatment with diethylcarbamazine（DEC) and albendazole while ivermectin is used against onchocerciasis. Repeat treatment is frequently required and this highlights the possibility of development of drug resistance. In addition, risk of adverse reactions following treatment excludes some patients from control programmes. Such circumstances urgently call for the development of complementary control measures such as vaccination.

DNA vaccines are novel type of subunit vaccine in which production of the immunizing antigen is induced in host cells after introduction of a plasmid or recombinant viral vector containing the gene that encodes the antigen. DNA vaccines are relatively simple and cheap to produce and their stability makes them particularly suitable for use in remote regions that lack the cold-chain storage facilities required of conventional vaccines. Filarial nematodes are tissue-dwelling parasites that survive for many years in immunocompetent hosts. It is proposed that this longevity is, in part, due to the capacity of the parasites to modulate potentially lethal Th2 responses of their hosts. Consequently, the efficiency of a filarial vaccine may depend on how well it circumvents filarial-driven immunosuppression.

To test this hypothesis, a series of DNA vaccines were developed and tested in the *Litomosoides sigmodontis*-mouse model of filarial infections. The *L. sigmodontis* Abundant Larval Transcript-1 (Ls-ALT) and Cysteine Protease Inhibitor (Ls-CPI) genes were cloned and genetically engineered to ablate their immunomodulatory properties by deleting the acid domain and by site mutation, respectively. In addition, the *L. sigmodontis* Venom Allergen Homologue (Ls-VAH) and Thioredoxin Peroxidase (Ls-TPX) were used in their

native forms as vaccine targets. To improve immunisation and antigen processing by the host, these parasite genes were fused to a DNA sequence encoding an antibody that specifically binds dendritic cell surface protein (αDEC205 single chain Fv). DNA plasmids carrying mutated forms and/or anti-DEC205 were then co-administered with plasmids encoding the Th2 promoting cytokine IL4, and/or antigen-presenting cell activating MIP1α and Flt3L.

Mice immunized with mutated forms (ADDALT and CPImu) of parasite antigens produced more specific antibody post-challenge and showed strongly increased lymphocyte stimulation above controls immunized with the native form. The immune response was further enhanced when plasmids encoding IL4, MIP1α, Flt3L and anti-DEC-205 forms were co-administered, resulting in production of a Th2/IgG1 phenotype. Significant reduction of worm burden (82.3%) was achieved by a cocktail vaccination which combined the ADDALT and CPImu candidates. Mice immunized with Ls-VAH and Ls-TPX DNA carried by αDEC205 elicited Th2-biased responses with up-regulated IgG1 and IgE antibodies as well as enhanced IL5, IL4, and IL13 and diminished IFNγ production compared to controls. The immune responses were further driven towards the Th2/IgG1 phenotype when Ls-VAH and Ls-TPX were injected with plasmids encoding ADDALT and CPImu and with the adjuvants Flt3L, MIP1α and IL4. This resulted in reduction in worm burden of 55.7% (cocktail vaccine containing Ls-VAH) and 41.6% (cocktail vaccine containing Ls-TPX) respectively in vaccinated animals.

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
ADDALT	Acidic domain deleted ALT
AEP	Asparaginyl endopeptidase
ALT	Abundant Larval Transcript
ANOVA	Analysis of variance
APC	Antigen-presenting cell
APOC	African Programme for Onchocerciasis Control
Asn	Asparagine
ASP	Ancylostoma secreted protein
Asp	Aspartic acid
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
cAMP	3'-5'-cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CPI	Cysteine proteases inhibitor
CPImu	Mutant of CPI2
CRISP	Cysteine rich secretory proteins
CTLA4	Cytotoxic T-lymphocyte antigen 4
CTL	Cytotoxic T lymphocytes
DCs	Dendritic cells
DEC-	DEC205
DEC	Diethylcarbamazine
dNTP	Deoxyribonucleotide triphosphate
EB	Ethidium bromide
E.coli	*Escherichia coli*

EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme linked immuno-sorbent assay
EN	Endemic normals
EP	Electroporation
FACS	Fluorescence-activated cell sorting
FcR	Fc receptor
FCS	Fetal calf serum
Flt3L	Fms-like tyrosine kinase 3-ligand
FOXP3	Forkhead box p3
g	gravitational force
GAGA3	GATA-binding factor 3
GITR	Glucocorticoid-induced tolerance receptor
GLM	Generalised linear models
GM-CSF	Granulocyte macrophage colony stimulating factor
ICAM	Intercellular adhesion molecule
IFNγ	Interferon gama
IgE	Immunoglobulin E
IgM	Immunoglobulin M
IL1	Interleukin 1
IL2	Interleukin 2
IL4	Interleukin 4
IL5	Interleukin 5
IL10	Interleukin 10
IL12	Interleukin 11
IL13	Interleukin 13
IL18	Interleukin 18
IL33	Interleukin 33
IL35	Interleukin 35

IL4Rα	Interleukin 4 receptor alpha
IL2Rα	Interleukin 2 receptor alpha
IL10R	Interleukin 10 receptor
IPTG	β-D-thiogalactopyranoside
IVC	Individually ventilated cages
L3	Third larval stage
L4	Forth larval stage
LAG3	Lymphocyte activation gene 3
LB	Luria-bertani broth
Lys	Lysine
Mf	Microfilariae
MHC	Major histocompatibility complex
Mins	Minutes
MIP1α	Macrophage inflammatory protein 1 alpha
MMLV	Moloney Murine Leukemia Virus
mRNA	Messager RNA
NKT	Natural killer T cell
OVA	Ovalbumin
OCP	Onchocerciasis Control Programme
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCL	Pleural cavity lavage
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease

RT-PCR	Reverse transcriptase PCR
RUNX	Runt-related transcription factor
s. c	Subcutaneously
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SOC	Super optimal broth with catabolite repression
SOCS1	Suppressor of cytokine signaling 1
SOD	Superoxide dismutase
STAT	Signal Transducer and Activator of Transcription
TAP	Transporter associated with antigen processing
TBE	Tris/Borate/EDTA
TBS	Tris buffered saline
TE	Tris/ EDTA
Th1	T helper cells 1
Th2	T helper cells 2
Th17	T helper cells 17
TGFβ	Transforming growth factor beta
TLR	Toll like receptor
TNFα	Tumor necrosis factor-alpha
TPX	Thioredoxin peroxidase
Treg	Regulatory T cell response
TSA	Thiol-specific antioxidant
UTR	Untranslated region

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Chapter 1-Introduction

1.1 Filarial diseases and treatment

Filariasis is the name given to a number of parasitic infections caused by filarial nematodes belong to the superfamily Filaroidia (Anderson RM, and May RM, 1992; Thomas BN, 2000). Eight filarial nematodes commonly infect humans (Table 1.1), cause a wide range of pathologies and can be generally classified into three groups: lymphatic filariasis, subcutaneous filariasis, and serous cavity filariasis.

Lymphatic filariasis is associated with the lymphatic system in which adult worms live and their physical obstruction of lymph flow can induce elephantiasis. The three principle species responsible for this disease are *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori.* Subcutaneous filariasis is associated with three major parasites: *Loa loa*, *Mansonella streptocerca* and *Onchocerca volvulus*. Adult worms of these species live the subcutaneous tissue of the skin. Serous cavity filariasis is caused by the worms of *Mansonella perstans* and *Mansonella ozzardi.* Their adult worms live in body cavities and can cause angioedema, pruritus, fever and serous cavities pain. All worms above distribute widely over the world and they can cause severe diseases in humans and animals (Table 1.1).

The most common human filariasises are lymphatic filariasis and onchocerciasis. They have similar life cycles, which consist of five developmental stages (L1, L2, L3, L4 and adult). After the male and female worms mate, the female gives birth to L1 (normally called microfilariae, Mf), which are ingested by the vector (mosquitoes in lymphatic filariasis; *Simulium.* spp in onchocerciasis) during a blood meal. The Mf moult and develop into the L2 and then L3 (infective) in the vector. The vector transmits the iL3 into the dermis layer of the skin. After about one year of development in the host, the larvae moult through two more stages and mature into adult to complete the full life cycle (Fig 1.1).

In the filaria life cycle, the infective larva (iL3) is a vulnerable stage of the parasite in the mammalian host in light of its size as well as biological requirements. It moults within about 7-10 days into L4. The potential of L3 larvae on induction of protective immunity against L3 challenge has been studied in both experimental and human filariasis. L3s have been found to secrete some important antigens which can elicit immune responses (Bleiss W *et al.* , 2002; Day KP *et al* , 1991b; Devaney E, Osborne J, 2000; Eisenbeiss WF *et al*., 1994; King CL, Nutman TB, 1991; Rajan TV *et al.*, 2002). However, targeting of other stages should not be neglected in the understanding of antifilarial immunity. Distinct reactivities against adult male worm antigens have also been identified in endemic normal humans (Turaga PS *et al.*, 2000), and it had been shown that putative immune responses could also be induced by Mf (Boussinesq M, 1991).

Filarial diseases have major social and economic impacts. In lymphatic filariasis, *W. bancrofti* counts for about 90% of about 120 million worldwide and *B. malayi* accounts for the rest and is mainly distributed in Southeast Asia. *B. timori* is found only in southeastern Indonesia. Onchocerciasis also affects widely and cause serious disease. It affects about 37 million people in 34 countries and is abundant in Africa, although there is small population in southern and Central America and the Yemen. (Addiss, 1998; Richards FO *et al.*, 1998; Saeftel M *et al.*, 2003).

Current control strategies mainly rely on mass drug treatment and a great deal of large scale international initiatives have been launched by WHO and the World Bank and supported by various charities with the goal of global elimination of filarial infections. These include: Global Alliance to Eliminate Lymphatic Filariasis (GAELF, http://www.filariasis.org/); Onchocerciasis Control Programme (OCP, ended in 2002, http://www.who.int/blindness/partnerships/onchocerciasis_OCP/en/); African Programme for Onchocerciasis Control (APOC, http://www.who.int/blindness/partnerships/APOC/en/), and Onchocerciasis Elimination Program for Americas (OEPA,

http://www.who.int/blindness/partnerships/onchocerciasis_oepa/en/). These programmes rely on a very limited number of drugs comprising diethylcarbamazine（DEC), albendazole and ivermectin.

In the case of lymphatic filariasis in Africa, ivermectin in combination with albendazole are used to control disease, while outside Africa it is a combination of DEC and albendazole (Table 1.2). DEC (6 mg/kg) kills adult worms while the combination with albendazole (400mg) likely to reduce the number of circulating Mf than DEC alone for longer periods. However, DEC cannot be used in Africa where is onchocerciasis co-endemic because of the risk of severe adverse reactions resulting from rapid death of great numbers of Mf in the skin and eyes inducing systemic inflammation that likely to be related to the release of *Wolbachia* (Cross HF *et al.*, 2001; Keiser PB *et al.*, 2002).

For control of onchocerciasis, annual or bi-annual treatment with ivermectin is under-use. Ivermectin kills Mf, however prolonged use can decrease fecundity despite adult worms are not killed (Basáñez MG *et al.*, 2008). However, in areas of Africa where the eye worm *L. loa* and *O. volvulus* is co-endemic, ivermectin cannot be used because of the risk of adverse reactions associated with death of *L. loa* Mf in and consequential neurological sequelae (Gardon J *et al.*, 1997; Twum-Danso NA, 2003). Furthermore, there is evidence of emergence of filarial resistance to ivermectin in humans and animals (Osei-Atweneboana MY *et al.*, 2007). These constraints accelerated the search for new antifilarial drugs. In this regard, the observations that many filarial nematodes including *Brugia. pahangi*, *Dirofilaria. immitis*, *B. malayi*, *W. bancrofti* and *O. volvulus* are the hosts of the endosymbiont bacteria of *Wolbachia* (Table1. 3, Bandi C *et al.*, 1999; Hoerauf A *et al.*, 2001; Townson S *et al.*, 2000; Rao R, 2002; Smith HL, 2000) provide us considerable prospects for alternate therapy through the use of antibiotics which target *Wolbachia*. This method may result in the adult worms and / or Mf killings. A study by Langworthy NG (2000) showed that oxytetracycline is macrofilaricidal effective for *O. ochengi*. Turner JD *et al* (2010) showed doxycycline

treatment was well tolerated by the onchocerciasis individuals. Similar results have been described by Hoerauf A *et al* (2000, 2001).

However, tetracyclines cannot be used on pregnant women or children who are below the age of 9 (Hoerauf A *et al.*, 2008). These constraints leave a proportion of subjects living in onchocerciasis endemic regions at risk from infection with *O. volvulus*. Such circumstances urgently need development of complementary measures to control filarial diseases. Taking together, these observations highlight the need of a safe and effective vaccine to prevent filariasis.

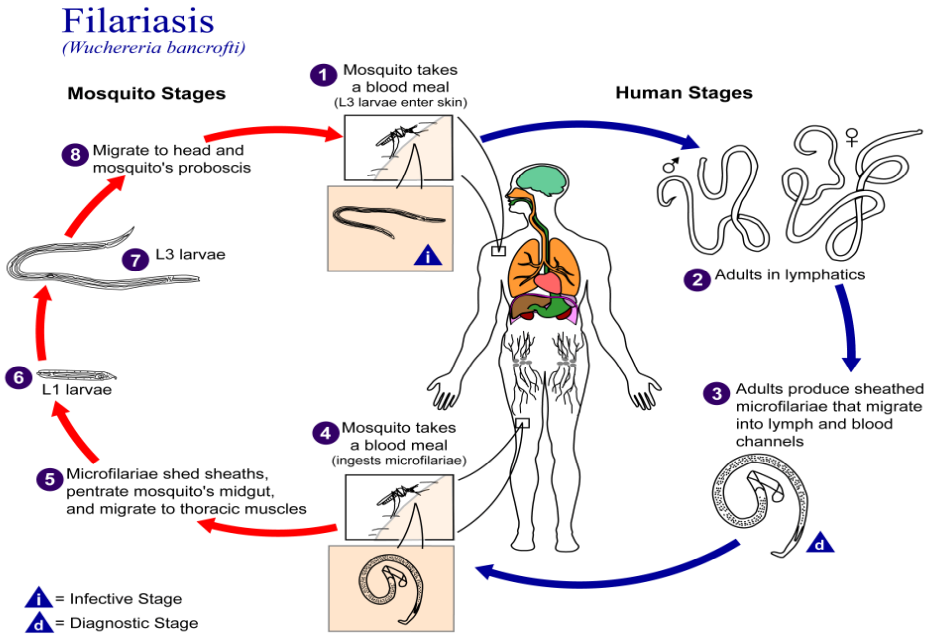

Fig. 1.1 Life cycle of filariae (Cited from http:// www. dpd. cdc. gov/dpdx)

Table 1.1 Filarial nematodes relevant to human diseases

Filarial Nematode	Transmitting Vector	Geographical Distribution	Relevant Disease	References
B. malayi	Mansonia & Anopheles	South & South east Asia	Lymphatic filariasis	Cheun HI *et al.*, 2009; Fischer P *et al.*, 2004;
B. timori	Anopheles	Lesser Sunda Islands of Indonesia	Timor filariasis	Fischer P *et al.*, 2004
L.loa	Deer fly/ Mango fly	Forest area of West and Central Africa (Cameroun, Gabon, Congo)	Loiasis Eye worm	Padgett JJ, Jacobsen KH. 2008
M. streptocerca	Midges	Rain forests of Africa	Streptocerciasis	Fischer P *et al.*, 1999 ; Walther M, Muller R. 2003
M. ozzardi	Midges Simulium	Tropical Americas	Mansonelliasis	Martins M *et al.*, 2010 ; Vaughan JA *et al.*, 2007
M. perstans	Culicoides	Africa &Tropical Americas	Mansonelliasis	Bregani ER *et al.*, 2007; Hoerauf A, 2009
O. volvulus	Simulium	Africa countries &isolated areas of South America	Onchocerciasis River blindness	Burnham G, 1998
W. bancrofti	Culex, Aedes and Anopheles mosquitoes	Africa, South America, Caribbean, South Asia and Pacific	Elephantiasis	Mak JW, 1987

Table 1.2 Recommended treatment strategies for mass drug administration (MDA),

(modified from Taylor MJ *et al.*, 2010)

Mass drug administration regions	**Diseases**		**Morbidity control**
	Lymphatic filariasis	Onchocerciasis	
Africa	Combination of ivermectin, 150μg/kg and albendazole 400mg, longer than 5 years	Ivermectin, 150μg/kg per year, 15-17 years	Lymphodema: hygiene, physiotherapy; Hydrocoele: doxycyline 200 mg per day, 6 weeks; Tropical pulmonary eosinophilia: doxycyline 200 mg per day, 4 weeks plus ivermectin, 150μg/kg twice a year; Onchocerciasis: Ivermectin, 150μg/kg twice a year until transmission stops; doxycyline 200 mg per day, 4 weeks plus ivermectin, 150μg/kg twice a year
Out of Africa	Combination of DEC, 6 mg/kg and albendazole, 400mg, longer than 5 years	Ivermectin, 150μg/kg twice a year until transmission stops	

Table 1.3 Distribution of *Wolbachia spp* in filarial and other nematodes

Nematodes with *Wolbachia spp* (Taylor MJ, Hoerauf A 1999*;* Taylor MJ *et al., 2005*)	**Nematodes without *Wolbachia spp*** (Bordenstein SR *et al.*, 2003; Duron O, Gavotte L. 2007; McNulty SN *et al.*, 2010)
B. malayi	*Onchocerca flexuosa*
Wu. bancrofti	*Setaria equine*
O. volvulus	*Acanthocheilonema viteae*
Onchocerca fasciata	*Dipetalonema setariosum*
B. pahangi	*Caenorhabditis elegans*
Litomosoides sigmodontis	*L. loa*
M. ozzardi	

1.2 Immunological research on filarial infections

In the past decades, immunological knowledge of protection against filariasis has been progressed. This provides clues to develop vaccines which also should be considered with any pathology associated with infection and the potential risk that this pathology may be induced by vaccination.

In 1987, Duke described the observation that even in hyper-endemic areas, there were still a small number of persons (1-5% of the population) who had no clinically and parasitologically sign of filarial infection (Word Health Organization, 1987), suggesting protective immunity can be induced following natural exposure. Analysis of Mf prevalence in Cameroon also revealed that not all persons living in endemic areas were infected with the filariae (Boussinesq M *et al.,* 1997) (Fig 1.2).

Similar observations were made in cattles with the parasite *Onchocerca. ochengi*. Tchakouté VL *et al* (2006)carried out longitudinal studies under conditions of either natural exposures or experimental infections in order to find out whether protective immunity against onchocerciasis exists. Results showed that cattles who were with putative immunity were less susceptible to heavy field challenge than age matched groups and naïve controls. Furthermore, melarsomine[*]-treated and patently infected cattles were fully susceptible. Also cattles immunized with iL3s were completely protected against experimental challenge, and this immunization also achieved against the prevention of severe and prolonged field challenge.

Two early studies of filariasis (Jachowski LA *et al.,* 1951; Leeuwin RS, 1962) showed that many individuals with microfilariamia moved from *W. bancrofti* endemic areas to nonendemic areas were never developed to chronic disease afterwards, indicating insistence might be induced when conditions were changed. Day KP (1991a) proposed a concept of “endemic normal” to describe the individuals who are Mf and symptoms or history of disease free despite life-long exposure to infection are processed. In support, Freedman DO

[*] Melarsomine is an arsenic-based drug that is effective against to microfilariae and adult heartworms and also can be used to treat some other nematodes.

et al (1989) identified 7 out of 459 amicrofilaremic adult people as endemic normals who were living in Mauke, Cool Islands. Furthermore, Steel C *et al* (1996) provided evidence that protective immunity to lymphatic filariasis existed and it might be mediated by T cells.

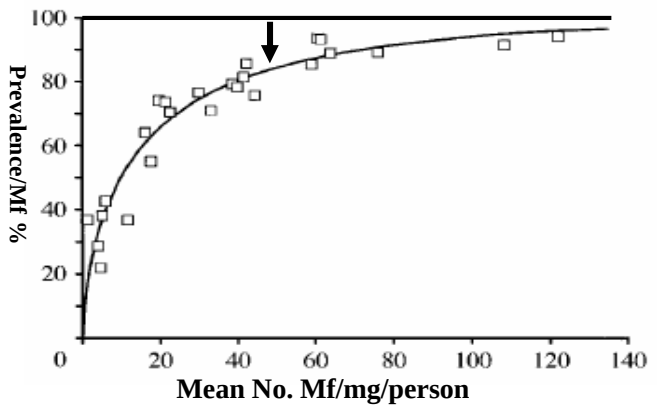

Fig. 1.2 The Mf prevalence (mean of Mf load per person) examed by Boussinesq in the community of 25 north Cameroonian villages (Boussinesq M, 1991, 1997) (sample sizes: 19 to 460 per village). The markers correspond to the age- and sex-adjusted Mf prevalence in the community using the whole sample (sizes:4576 people) as the reference (Figure is modified from (Basáñez MG, Boussinesq M, 1999))

Evidence of protective immunity also comes from onchocerciasis. Ward DJ *et al* (1988) described differences of parasite-specific T cell subpopulations between infected and putative immune individuals. Results demonstrated that these differences might be correlated with the development and maintainance of protective immunity to *O. volvulus* infection. Nutman TB *et al* (1991) compared antibody specificity in onchocerciasis patients to define potential antigens of antibody responses using sera from 12 persons without onchocerciasis and 16 people with *O. volvulus* infection. They found there were no differences in antigen recognition using *O. volvulus* adult antigens between the putative immune individuals and infected subjects. However, IgG from the putative immune individuals preferentially recognized 45- to 50-kDa and 22-kDa L3 antigen when *O. lienalis* L3 antigens were used.

Therefore, based on the evidence from human and cattle studies, protective immune responses do exist and might be associated with Th2 responses. This is supported by work with experimental models that demonstrate the Th2 immune responses are dominant during filarial infections. Early experiments of *B. pahangi* in cats provided clear evidence of

acquired resistance against reinfection with L3s, and this resistance mainly targeted to L3 larvae, with destruction of the majority in 1 day post infection (Denham DA *et al.*, 1983), suggesting protective immune responses can be induced by L3 larvae. Subcutaneous infection of BALB/c mice with *B. pahangi* L3 (Osborne J *et al.*, 1996) resulted in production of a strong Th2 response characterized by increased IL4, IL5, and IL10 and parasite-specific IgG1 and IgE. Similarly, Th2 cytokines IL4, IL5, IL10 and IL13 were grown up in gerbils after a primary subcutaneous infection with *B. pahangi.* Also a peak of peripheral eosinophilia was presented at 4 weeks after infection in these animals (Chirgwin SR *et al.*, 2005). Osborne J and Devaney E (1998) described that in the draining lymph node of L3- but not Mf-infected mice IL4 transcription 4 days post infection (p.i) peaked, at this stage, IL4 was the only detectable cytokine. In contrast, high mRNA levels of IFNγ were induced by Mf at day 4 p.i. without production of IL4 or IL10. Bancroft AJ *et al* (1994) described a strong Th2 bias protective immune response to a challenge infection was induced by immunization subcutaneously (s.c.) with iL3s of *B. pahangi* in BALB/c mice and this resulted in a 75-100% parasite burden reduction 6 days post challenge. Furthermore, Paciorkowski N *et al* (2003) found that B cells in pleural cavity were required for clearance of primary and challenge infections with *B. pahangi* L3s and the early productions of Th2-type cytokines.

Th2 biased immune responses can also be induced in other experimental models. For example, studies on *D. immitis* showed that microfilariaemic dogs had high level of IL4 and IL10 in contrast to their marginal expression in amicrofilariemic dogs. It was also showed that Mf positive status was associated with high level of IgG1 against worm antigens (Morchón R *et al.*, 2007). Marcos-Atxutegi C *et al* (2003) showed that the Th2 type responses provided protection against *D. immitis* larval challenge. High levels of IFNγ, IL4, IgG1 and IgE were produced in BALB/c mice inoculated with soluble antigens from *D. immitis* L3s or its adult worms. The cytokine response was similar to other filarial infections, with responses biased towards Th2. Moreover, Tezuka H *et al* (2003) evaluated the ability of a *D. immitis* protein, which presents as 15-kDa monomer and 30-kDa dimmer, to induce

Th1/Th2 responses. Results indicated that the monomer rather than the dimer protein had a greater capability to stimulate IgE and was associated with both B cell activation and IL4 production.

Studies using the *L. sigmodontis* mouse model provided further evidence that Th2 immune responses contribute to protection against filarial infection. During chronic and potent infections of *L. sigmodontis*, susceptible BALB/c mice showed a strong Th2 response with high levels of IL4, IL5 and IL10 58 days post infection while in resistant B10.D2 mice had no none (Maréchal P *et al.*, 1997). Additionally, evidence of eosinophils on killing of helminths by eosinophil peroxidase (EPO) and major basic protein (MBP) has been shown to be part of the defense mechanism against filarial nematodes, with support that lack of EPO or MBP impaired the defense ability against murine filarial infection (Specht S *et al.*, 2006).

More evidence of Th2 responses induced by nematode infection comes from work with: 1), *B. malayi* in BALB/c mice. For instance, acceleration of live parasites elimination was found in mice immunized with soluble microfilarial antigens. Results also showed the clearance of Mf was associated with local eosinophilia and increased serum IgE levels. Th2 cytokines in response to *B. malayi* antigens were produced by $CD4^+$ cells from the site of parasite challenge (Pearlman E *et al.*,1993). 2), Intestinal *Trichinella spiralis* and *Nippostrongylus brasiliensis* in rodents. Studies have shown that Th2 rather than Th1 responses mediated resistance to the nematodes. Also Th2 cytokines were associated with worm killing and specific cellular responses by study using neutralizing antibodies and genetically engineered mice (Mahida YR, 2003).

Development of Th2 responses may also be a consequence of immunomodulation by the parasites (Tawill S *et al.*, 2004). For example, Th2 responses in mice could be driven by the excreted/ secreted antigens from *N. brasiliensis* without requirement of infection (Holland MJ *et al.*,2000). Th2 differentiation could be triggered by the *A. viteae* secreted phosphorylcholine-bearing protein ES-62 (Whelan M *et al.*, 2000). Carbohydrates also mediated the induction of Th2 responses, with the examples of soluble extracts from *B.*

malayi and schistosome soluble egg antigen (SEA) (Pearce EJ, MacDonald AS, 2002; Tawill S *et al.,* 2004). Similarly, proteins such as proteases could also be Th2-trigers (Sokol CL *et al.,* 2007). According to Lawrence RA *et al* (1994), Th2 cell differentiation could be driven by adult females rather than Mf. The Th2-biased responses induced in mice implanted with *B. malayi* might be driven by alternatively activated macrophage (Loke P, *et al.,* 2000; Rodríguez-Sosa M, *et al.,* 2002). Also T h2 responses with the high production of IL4, IL5, IL6, IL9 and IL13 followed by IgG, IgE could be induced by Eosinophils and mast cells which are early sources of IL4 (Maizels RM *et al.,* 2004).

However, the conventional Th1/Th2 dichotomy does not explain all the clinical and parasitological manifestations caused by filarial infections. In the case of onchocerciasis, the asymptomatic individuals harbor abundant Mf in tissue but no overt responses to their presence (Mackenzie CD *et al.,* 1985). Hyper-responsive individuals with pathology were classified into another group characterised with Mf-negative (some patients are symptomatic microfilaridermic) but pathology-positive. In this sense, the presence of Mf appears necessary for the pathology resulted from the death of Mf in tissue (Connor DH *et al.,* 1985; Mackenzie CD *et al.,* 1985). In contrast, individuals suffering from severe skin pathology (*Sowda*) showed reduced Mf in the skin, increased cell-mediated responses and elevated IgE and IgG (Brattig NW *et al.,* 1987; Lucius R *et al.,* 1986).

Similar clinical manifestations have also been observed in lymphatic filariasis in humans (Mahanty S *et al.,* 1996; Ottesen EA *et al.,* 1977; Sasisekhar B *et al.,* 2005; Steel C *et al.,* 1994) and animal models with the presence of lymphocytes in the bloodstream (Harnett W, Harnett MM, 2008; Taylor MD *et al.,* 2006). Studies in onchocerciasis have shown that hypo-responsive individuals have high levels of IgG4, IgE and reduced levels of IFNγ whereas hyper-responsive individuals have high levels of IgG1, 2, 3 and elevated IFNγ, indicating that more complicated mechanisms apart from both Th1 and Th2 responses may be involved (King CL *et al.,* 1993; Maizels RM *et al.,* 1995).

The Th1/Th2 paradigm has been used as a framework of examination of immune responses in humans and mice for many years, however, criticism for over-simplifying the real complexity of infections *in vivo* was rising (Allen JE, Maizels RM, 1997; Maizels RM *et al.*,1999) (Table 1.4, Fig. 1.3, 1.4). The discovery of regulatory T cells (Tregs) makes the picture of immune response much clearer. There are two types of $CD4^+$ Tregs, 'natural' Tregs (nTregs) and induced Tregs (iTregs). nTregs develop in the thymus, while iTregs develop from $CD4^+$ T cells following antigenic stimulation under different circumstances in the periphery (Workman CJ *et al.,* 2009). There are three Treg phenotypes, Tr1, Th3 and $CD4^+CD25^+$ cells. Tr1 is induced by IL10 (Groux H, 1997; Vieira PL, 2004), while Th3 is induced by TGFβ (Weiner HL, 2001). $CD4^+CD25^+$ cells (nTregs) were first described by Sakkguchi in 1995 (Sakaguchi S, 1995) then fork head box p3 (Foxp3) was found to be expressed by both nTregs and iTregs in humans and mice (Fontenot JD, 2005; Sakaguchi S , 2004). Although there are many markers of Tregs (Table 1. 5), the Foxp3 is more generally accepted as the marker of nTreg development and functions even there are reports showed that not all $Foxp3^+$ human T cells are suppressive (Ziegler SF, 2006). Indeed, it has been shown that regulatory capacity is needed when conventional T cells are induced to express Foxp3 via retroviral transduction *in vivo* and *in vitro* (Fontenot JD *et al.,* 2003; Hori S *et al.,* 2003).

Tregs can suppress both Th1 and Th2 activities (Fig. 1.3) and affect the outcome of infection with protozoan (Belkaid Y *et al.,* 2002; Hisaeda H *et al.,* 2004), viral (Iwashiro M *et al.,* 2001) and bacterial (Kullberg MC *et al., 2*002; McGuirk P *et al.,* 2002). The precise mechanisms of Treg cell function have not been clear. Significant progress has however been made in identification of their roles in filariasis in the past years. Although Foxp3 has proved to be an invaluable marker of mouse Treg cells, expression of Foxp3 alone is not enough for regulatory function. Doetze A *et al* (2000) found that *O. volvulus* (Ov)-specific hypo-responsiveness was mediated by IL10 and TGFβ. Significantly greater IL10 produced by the PBMC from individuals with generalized onchocerciasis (GEO) could be reversed by

anti-IL10 and anti-TGFβ antibodies treatment. Satoguina J *et al* (2002) showed that although patients who had little dermatitis demonstrated millions of onchocerca worms in the skin, Tr1 cells could be obtained and could induce an increased CTLA-4 after stimulation and inhibit other T cells. In *L. sigmodontis* model, Treg function (Fontenot JD *et al.*, 2003), the increased IL10 and TGFβ are associated with an early increase of Foxp3 mRNA in the draining lymph nodes. Moreover, the levels of CTLA-4 and GITR increase despite IL5 decreases during infections. It has been demonstrated that removal of Tregs activities could reverse hypo-responsiveness and lead to filarial parasite clearance by anti-CD25 and-GITR treatment(Taylor MD *et al.*, 2007), furthermore, the protective immunity *in vivo* was inhibited by the CTLA-4 and $CD4^{+}CD25^{+}$ regulatory T cells. Recent research showed that the early recruitment of nTregs determined outcome of filarial L3 infection (Taylor MD *et al.*, 2009).

Recent researches suggested more factors are involved in Treg functions. Collison LW *et al* (2007) IL35 might be specifically produced by Treg cells and required for maximal suppressive activity of Tregs. Studies from Bopp T *et al* (2007) showed that nTreg cell-mediated suppression and membrane traverse via gap junctions are associated with cyclic adenosine monophosphate (cAMP). Huang AY *et a*l (1996) showed that Tregs populations are marked by lymphocyte activation gene 3 (LAG-3). Studies from Kitoh A *et al* (2009) indicated that *in vivo* Treg cell inhibition and optimal expression of FoxP3 gene are mediated by the Runt-related transcription factors (Runx1)-core-binding factor-beta (Cbfβ) heterodimer. Additionally, data from Klunker S *et al* (2009) showed that TGFβ-induced Foxp3 expression during iTreg cell differentiation is linked by Runx transcription factors. Further, the Eos gene plays an essential role in Foxp3-dependent gene silence of Tregs. Silencing of Eos in Tregs gets rid of the suppressive abilities to immune responses (Fan Pan *et al.*, 2009).

Table 1. 4. Summary of T, B-cell response of different onchocerciasis patients

Patient group	Hypo-responsive response	Hyper-responsive response	Putative immune response	Refs
T-cell	Th2, Th3/Treg	Th2++	Th1, Th2++	Brattig N *et al.*, 1997; Elson LH *et al*, 1995; Soboslay *et al.*, 1997; Ward
B-cell	IgG1+, IgG3, IgG4 ++, IgE+++	IgG1++, IgG3, IgG4 ++, IgE+++	IgG1, IgG3, IgG4 IgE+	Doetze *A et al.*, 2000; Elson LH *et al.*, 1995; Ward DJ *et al.*, 1988
Effector response	Eosinophils + Serum+	Eosinophils + Serum++	Eosinophils Serum -	Bradley JE *et al.*, 1996; Brattig N *et al.*, 1997; Brattig NW *et al.*, 1994
Clinical picture	Disease,not protected	Disease, and Mf killed	No disease and protected	Brattig NW *et al.*, 1994; Tischendorf FW *et al.*,1996

Abbreciations: Th1, T helper 1 cells; Th2, T helper 2 cells; Th3, T helper 3 cells; Treg, T regulatory cells; Mf, microfilaria; Ig, immunologlobulin; +, represents intensity of response or number of cells.

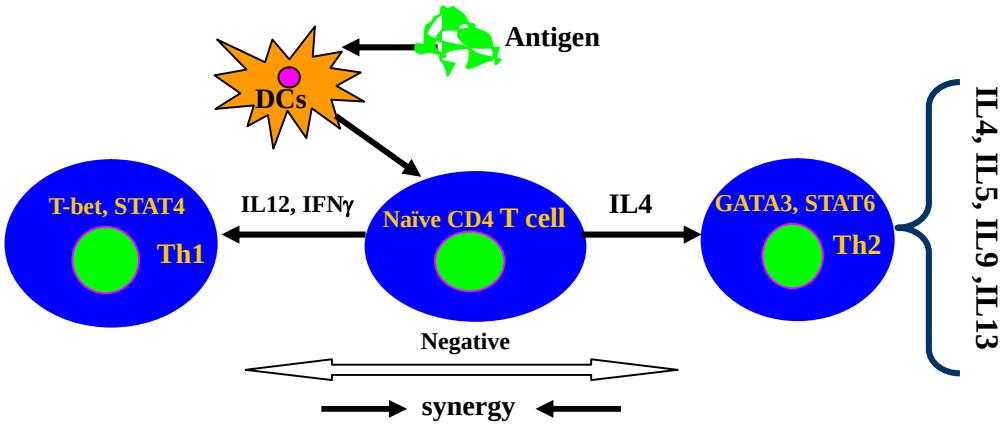

Fig. 1.3 Th1/Th2 paradigm and differentiation pathway. Th2 and Th1 responses generally active antagonistically, however, the synergic actions have also been described.

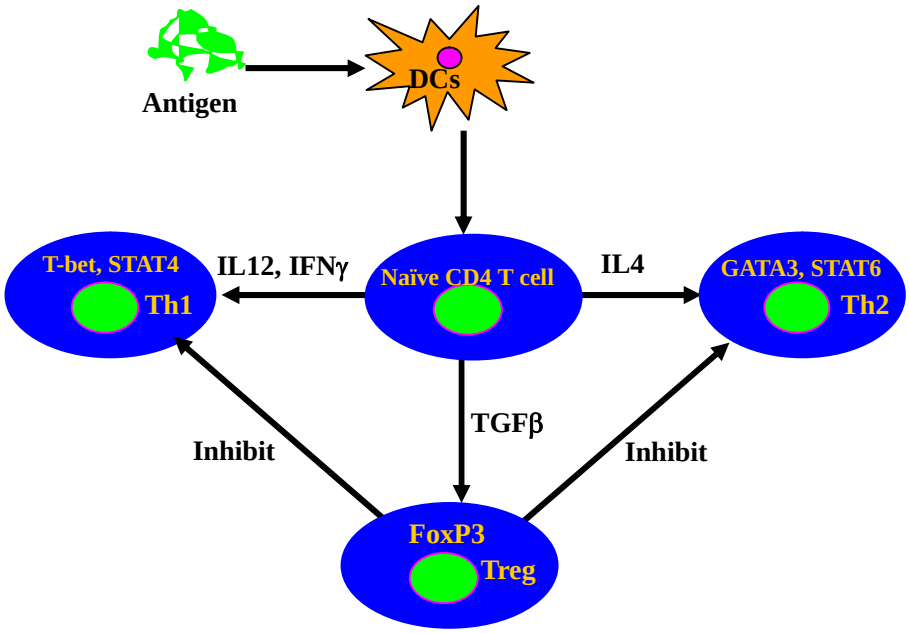

Fig. 1.4 Relationship between Treg, Th1 and Th2 response

Table 1.5 Molecular markers associated with Th1, Th2, Th17 and Tregs. (Cells associated with these responses are not concern in present study)

Response	Molecules	Function	Expression	References
Th1	IFNγ	activates macrophages, CTLs and NK cells; drives anti-viral signals in target cells	Activated T cells; NK cells	Reviewed in (Saito *S et al.*, 2010)
Th2	IL4	provides positive reinforcement signal to drive Th2 cells expansion ; as Th2 effector	Activated T cells; APCs	Reviewed in (Gaffen SL. *et al.*, 2008)
	IL5	as Th2 effector	Activated T cells; APCs	Reviewed in (Gaffen SL *et al.*, 2008)
	IL13	as Th2 effector	Activated T cells; APCs	Reviewed in (Gaffen SL *et al.*, 2008)
Th17	IL17A	induction of inflammation	Activated T cells; Th17 cells	Reviewed in (Richard M and Locksley, 2009)
	IL17F	induction of inflammation	Activated T cells; Th17 cells	Reviewed in (Richard M and Locksley, 2009)
Tregs	CD25	Alpha subunit of IL2R	nTregs; Activated T cells	Reviewed in (Ohkura N, 2010)
	Foxp3	Transcription factor	Tregs; other cell types	Reviewed in (Workman CJ *et al.*, 2009)
	CTLA-4	Negative co-stimulatory molecule; can bind to B7 molecules	Tregs; Activated T cells	Reviewed in (Sansom DM, Walker LS, 2006)
	GITR	Positive co-stimulatory molecule; can bind to GITRL	Tregs; Activated T cells	Reviewed in (Shevach EM, Stephens GL, 2006)
	IL10	Inhibits Th1 cytokines; promote Th2 cytokines; binds to IL10R	Tregs; Th2 cells; APCs	Reviewed in (Moore KW *et al.*, 2001)
	TGFβ	Suppressive cytokine; binds to TGFβR	Tregs; B cells	Reviewed in (Xu L *et al.*, 2010)

Moreover, Th2 responses can be also induced by irradiated L3 vaccination. For instance, a study on *L. sigmodontis*- BALB/c mice model showed the high levels of specific IgM and IgG subclasses (IgG1, IgG2a and IgG3), high specific IL5 from spleen cells *in vitro* and a high number of eosinophils in subcutaneous tissue contribute to protection (Le Goff L *et al.,* 2000). Another study showed a significant but partial protection up to 5 months post vaccination with iL3s was achieved. However, this immunity did not extend to the next life stages (Babayan SA *et al.*, 2006). More importantly, iL3 vaccination in *L. sigmodontis* -BALB/c mice model showed a type 2 bias immune response and maintained its efficacy even repeated parasite challenges were given (Hübner MP *et al.,* 2010).

Th2 biased responses can also provide protection against *O. volvulus* in mice based on the following facts. a) IL5 instead of IFNγ was detected in diffusion chambers from vaccinated mice (Lange AM *et al.*, 1994). b) Levels of IL4 and IL5 increased when spleen cell cultures were stimulated wih *O. volvulus* antigen (Taylor MJ *et al.*, 1994). c) Significantly decreased protective responses of iL3 vaccination were demonstrated by treatment witheither ant- IL5 or -IL4 mAb. and d) No protection was obtained by immunization in IL4 defect mice (Johnson EH *et al.*, 1998).

Similar observations were made by Girod N *et al* (2003) in BALB/c mice immunized with iL3 of *Necator americanus*. Further support comes from the work of Ricardo T *et al* (2006) on dogs that showed high level of antibodies, strong PBMC proliferation to L3 antigens and production of IL4 were evoked by iL3vaccination. Similar observations have been obtained from experiments with *A. viteae* in *Meriones unguiculatus*, *Mastomys coucha* and *Mesocricetus auratus* (Schrempf-Eppstrin B *et al.,* 1997), *L. loa* in *Mandrillus sphinx* (Akue JP *et al.*, 2003; Ungeheuer M *et al.*, 2000), *Nematodirus battus* in lambs (Winter MD *et al.*, 2000)and *Ancylostoma caninum* in dogs (Boag PR *et al.*, 2003; Fujiwara RT *et al*., 2006), mice and hamsters (Jian X *et al.*, 2006).

1.3 Vaccination and DNA vaccine

1.3.1 General vaccination

Success vaccinations against infectious diseases have been achieved for over 200 years. Indeed, vaccination is considered to be the most cost-effective method to prevent human disease and reduce animal suffering and economic losses. However, even with these successes on vaccination, infectious diseases are still economical significant on reduction of productivity and animal death. The discovery of biotechnologies, genomics, proteomics together with understanding of immune responses to pathogens and pathogenesis provide us excellent opportunities to develop more efficient and safer vaccines.

Although vaccination has became commonplace against many bacterial and viral infections (Table 1. 6), development of vaccines against helminth infection has been very slow. Indeed, the only commercially available vaccine against helminth infection is that used for *Dictyocaulus viviparus*, the cattle lungworm. This vaccine comprised iL3 larvae and could obtain 95% reduction in adult worm burdens when calves were vaccinated with 1000 iL3 larvae and challenged orally with 3000 to 4000 larvae (Bain RK, Urquhart GM, 1988).

The capacity of iL3 to evoke protective immunity has also been demonstrated in the hookworm (*N. americanus* and *A. caninum*) in mice and hamsters (Jian X *et al.*, 2006), as well as *A. viteae* in three rodents (*M. unguiculatus, M. coucha, and M. auratus* (Schrempf-Eppstrin B *et al.*, 1997)

In the case of *L. sigmodontis* in mice, more than 50% protection was induced by three inoculations with 25 iL3s after challenge infection. Th2 responses contributed to this protective immunity and the enhanced L3 killing in vaccinated challenged mice appeared to be associated to eosinophils (Abraham D *et al.*, 2002; Le Goff L *et al.*, 2000; Maréchal P *et al.*, 1997; Storey DM, Al-Mukhtar AS, 1982). An important feature of immune responses

stimulated by *L. sigmodontis* is the key role played by Treg cells (Dittrich AM *et al.*, 2008; Taylor MD *et al.*, 2007). This is a critical observation for the design of vaccines and furthermore, responses parallel those seen in human onchocerciasis (Table 1.5).

However, although the use of iL3 larvae in veterinary medicine demonstrated the efficacy and feasibility of iL3 vaccines, it is impossible that such vaccines would be either practical or acceptable for human use, because of the impossibility of producing enough L3s and the lack of acceptability by the public. The consequence is a focus on development of dead vaccines, although use of attenuated viruses or bacteria as the carrier and delivery systems remain as options.

There are two main approaches for development of dead vaccines including recombinant protein and DNA vaccination. In the case of recombinant protein, the hepatitis B (Zhu FC *et al.*, 2010) and human papilloma virus (HPV, Albarran Y *et al.*, 2007) vaccines have demonstrated their efficacy. In the case of eukaryote parasites, recombinant vaccines against the animal stage of*Echinococcus* spp (Heath DD *et al*., 2003), *Taenia ovis* (Rothel JS *et al.*, 1997) and ticks (Prudencio CR *et al.*, 2010) have also been developed. Moreover, the malaria RTS vaccine against *Plasmodium falciparum* showed promise after phase II trials in children (Sacarlal J *et al.*, 2008; Barbosa A *et al.*, 2009 ; Lell B *et al.*, 2009).

DNA vaccines, although few are registered for commercial use, exhibit many advantages. First, humoral and cellular immune responses can be achieved in animal models at low dosages of DNA. In addition, they are safer than live attenuated vaccines (Meeusen EN *et al.*, 2007). Finally, DNA vaccines are more stable than conventional vaccines, this significantly enhances vaccine storage and mobility (Table1.6).

DNA vaccine development against eukaryote parasites is progressing. DNA-based vaccinations have been reported against *Trypanosoma cruzi* (Limon-Flores AY *et al.*,2010),

Toxoplasma gondii (Li B *et al.*, 2010), *Schistosoma japonicum* (Zhu Y *et al.*,2010), *Leishmania* (Ahmed SB *et al.*, 2009) and *B. malayi* (Ramachandran S *et al.*, 2004; Thirugnanam S *et al*., 2007). Moreover, “prime-boost” strategies have been applied in malaria with some success. The mice that were primed with plasmid DNA which encodes *Plasmodium yoelii* circumsporozoite surface protein (PyCSP) were boosted with a recombinant vaccinia virus encoding the same protein, and significantly higher levels of antibodies, IFNγ production and CTL activities than the mice that were immunized and boosted with plasmid DNA alone were obtained (Sedegah M *et al.*, 1998). This protective responses can be further augmented by a prime of a mixtureof plasmids encoding PyCSP and murine GM-CSF and a boost with recombinant vaccinia virus (Sedegah M *et al.*, 2000). An effective prime-boost strategy for the *Plasmodium. knowlesi* model has also been demonstrated (Rogers Wo *et al.*, 2001). Rhesus monkeys were primed with a DNA vaccine consisting the merozoite surface protein 1 (PkMSP1p42), the apical merozoite surface protein 1 (PkAMA1), sporozoite surface protein 2 (PkSSP2) and circumsporozoite surface protein (PkCSP), then were boosted with a recombinant canarypox virus encoding all these four antigens. All these immunized monkeys developed IFNγ-inducing T cell responses against peptides from PkCSP as well as humoral responses against sporozoites and infected erythrocytes. Moreover, “prime-boost” vaccination regimens against *P. falciparum* which involve recombinant simian adenoviruses and plasmid DNA have recently been assessed as priming regents in Adenovirus-MVA regimens in both phase I and phase IIa trials in United Kingdom. These regimens showed better immunogenicities and efficacies than earlier vaccinia virus Ankara regimens (Moorthy VS *et al.*, 2004;Gilbert SC *et al.*, 2006; Hutchings CL *et al.*, 2005;Webster DP *et al.*, 2005;Hill AV *et al.*, 2010).

Table 1. 6. Comparison of several types of vaccines (Modified from Meeusen EN *et al.*, 2007).

Type of vaccine	Commercial products	Advantages	Disadvantages
Live vaccine	Measles, mumps, rubella (German measles), polio (Sabin vaccine) and chicken pox; Chicken coccidiosis; *Theileria parva;* *T.gondii* (Toxovax) *Dictyocaulus* (Dictol®);	Produces strong immune responses; provides life-long immunity.	Not safe for subjects with compromised immune systems. Needs refrigeration to maintain potent.
Killed vaccine	Cholera, flu, hepatitis A, rabies, polio (Salk vaccine); *Babesia canis;*	Safe for subjects with compromised immune systems. Easily stored and transported; No refrigeration requirement.	Usually requires boosts every few years to keep effective.
Subunit vaccine	Hepatitis B; Ticks: recombinant antigen (TickGARD®); *Eimeria. maxima* (CoxAbic)	Lower chances of adverse reactions.	Researches can be time-consuming and difficult.
Conjugate Vaccine	Haemophilus influenzae B (or Hib) and pneumococcal vaccine	Safe for subjects with immuno-compromised systems.	Usually requires boosts every few years to keep effective.
Synthetic Peptides	Foot and mouth disease vaccine	Easier quality control; less toxic; safer; feasible even agents cannot be cultivated.	Less immunogenic; requires adjuvants, needs primary and boosters.
DNA vaccine	IHN virus ; West Nile virus;	Produced by simple genetic techniques; can be used for multiple diseases in one injection; extremely stable.	Not effective for all agents; possible to induce antibody response against DNA and tolerance to the antigen (protein) produced;

1.3.2 Mechanism of DNA vaccine presentation

DNA vaccines were first invented by Wolff and colleagues in 1990 (Wolff JA *et al.*, 1990),

and were developed from “failed” gene therapy experiments (Tang D *et al.*, 1992). DNA vaccines are based on the possibility of induction of both humoral and cellular immunities against antigens. DNA-based immunization is also an attractive strategy in the prevention of infectious diseases.

DNA vaccines are made up of a small circular plasmid which has been genetically engineered to produce one or multiple specific antigens from a pathogen. DNA vaccines are commonly delivered by simple intramuscular injection (i.m), in muscular tissue the expressed proteins form depots of antigens and stimulate both MHC II and MHC I-restricted antigen presentation pathways. In the case of MHC I-restricted cytotoxic T lymphocytes (CTLs), they may be induced by directly transfected somatic cells (keratinocytes, myocytes or any MHC II-negative cells) or by professional antigen-presention cells (APCs), or by a cross-priming method (Donnelly JJ *et al.*,1997; Sharma AK, Khuller GK, 2001). The process might be as follows: when the DNA vaccine reaches cell nucleus, the transcription is started then translation of the encoded protein is processed on cytosolic ribosomes. The synthesized proteins are processed and released in the proteosome complex. Then they are transported to the endoplasmic reticulum via the membrane transporter complex TAP-1 and TAP-2. Afterwards, the transported peptides are bound to MHC I /MHC II molecules which locate inside the endoplasmic reticulum (Huang AY *et al*., 1996; Rock KL, Goldberg AL, 1999). Then, these bound peptides migrate to the cytoplasmic membrane and are present to the $CD8^+$/$CD4^+$ T cells (Huygen K, 2005) (Fig. 1.5).

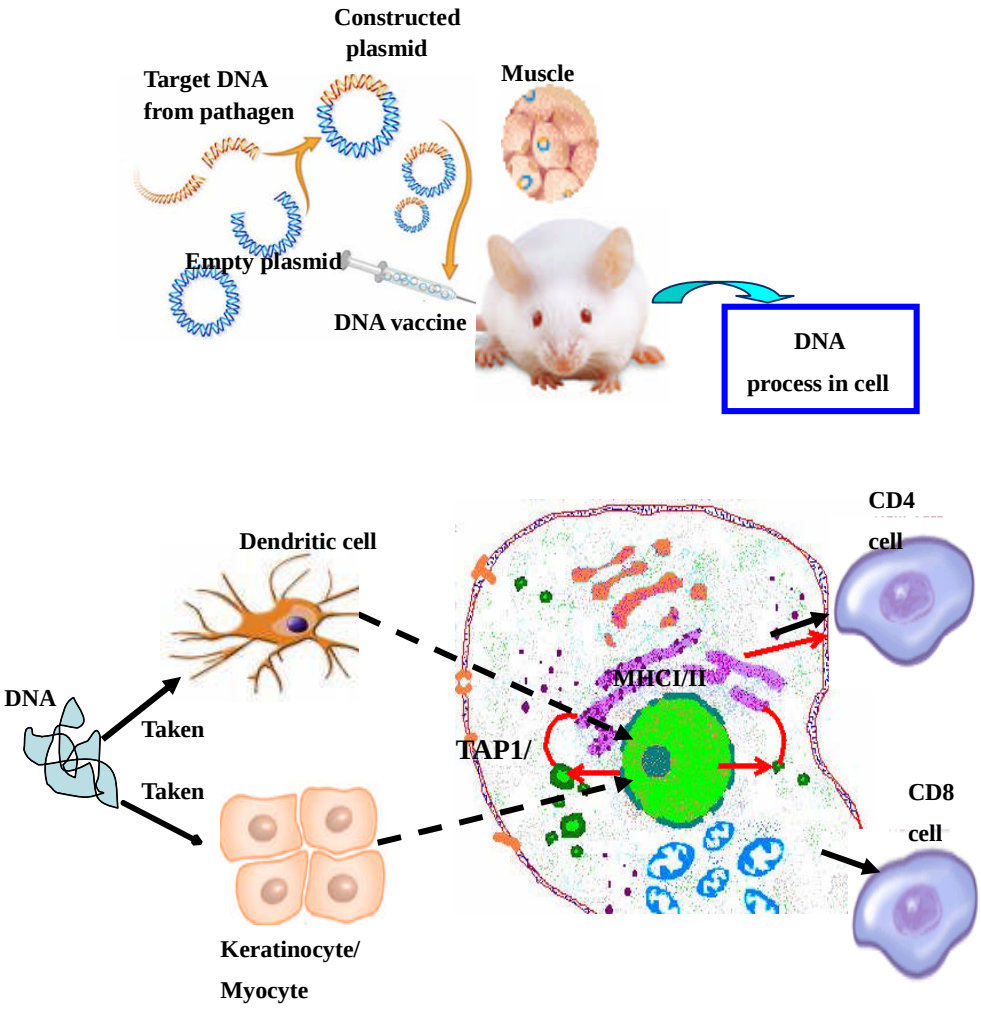

Fig. 1.5 Routes of antigen presentation of DNA vaccine.

1.3.3 Roles of dendritic cells (DCs) in DNA-based immune induction

It has been well documented that APCs are the key stimulators of immunity and functions because they build immunological bridges between somatic cells and T cells. They can also interact with antigens in the site of delivery and secondary lymphoid organs. DCs have been identified to distribute in all the lymphoid and connective tissues. They can present antigens to naïve T cells within secondary lymphoid tissues. Also DCs are potent activators of naïve T cells and play three distinct roles in priming the immune system to DNA vaccine antigen: 1), MHC II-restricted presentation of antigen, 2), MHC I-restricted presentation, and 3),

MHC I-restricted “cross-presentation” of vaccine antigen. It is likely that each role is essential in DNA vaccination and it is not enough for one of them to elicit potent humoral and cellular responses.

The first function of DCs in immunity induction relies on their phagocytic ability to capture and process secreted vaccine antigen expressed by plasmid-transfected cells. When DCs receive the proper signal, they induce and up-regulate co-stimulatory molecules such as B7 and then interact with antigen-specific Th cells. These Th cells secrete Th2 cytokines in subsequent pathways. Although antigen secretion from DNA vaccine might aid to increase the humoral response, it might not induce CTLs, and thereby this function of DCs cannot act independently of the other two roles in the induction of immunity.

An additional mechanism of immunity induction depends on the transfection of DCs at the site of administration. A small amount of APCs express, process, and present the vaccine antigen then migrate to draining lymph nodes (DLN) in which they interact with naïve T cells. Casares S *et al* (1997) showed A/PR/8/34 influenza peptide encoded plasmid DNA has been isolated and identified directly from DCs in the local lymph nodes and skin following i.m injection. Condon C *et al* (1996) described that green fluorescent protein (GFP)-expressing Langerhans cells (LCs) were identified in DLN after GFP-encoding plasmid injection using gene gun, this supports the idea that skin LCs are directly transfected. Chattergoon MA *et al* (1998) have shown that DCs can be directly transfected and migrate to the regional lymph nodes, and can be found in the blood stream following i.m injection. When $CD4^+$ T cells are activated in this manner, they can migrate to the spleen (Akbari O *et al.*, 1999b).

The final mechanism of DC immune induction is associated with the “cross-presentation” of vaccine antigen to T cells in a MHC I-restricted manner. Ulmer JB *et al* (Ulmer JB, 1996; Ulmer JB, 1997) reported that the stably transfected myoblasts chimeric mice had the

capability to induce protective antibody and CTL responses. These responses are restricted to the MHC of the APCs from bone morrow, and are distinguished from the immune responses induced by plasmid vaccination alone. Also successful cross-priming of antigens needs a "danger" signal or a pro-inflammatory condition which is produced by opsonization and cellular apoptosis.

Therefore, strategies to optimize antigen presentation by dendritic cells might be a rational effective approach to DNA vaccine design.

1.3.4 DNA vaccine delivery and electroporation

Various strategies have been tested to overcome early weak performances of DNA vaccination in primates. These approaches included particle-mediated delivery and gene gun. However, these are only advantageous with low doses of DNA (Fuller DH *et al*., 2006). For the i.m route, a main reason for the weak performance in large animals has now been uncovered by the application of electroporation. It is already clear that the volume of injection is crucial in this approach. Methods used to enhance the efficiency of DNA delivery can be classified into three general types: mechanical, chemical and biological method. Needle injection, gene gun and needle-free jet injection are the main mechanical DNA delivery approaches. Needle injection needs much more DNA than gene gun, while needle-free jet injection is more practical in laboratory animals than in clinical humans and livestock animals.

In the case of chemical approaches, there are five general types of delivery systems. 1), DNA mixed with cationic polymers including poly-L-lysine, protamine sulfate, polyethyleneimine, chitosan, polyethylene glycol, poly-(D, L-lactide-co-glycolide). 2), DNA with artificial cationic lipids or lipopolyamines. 3), DNA with artificial anionic lipids through electrostatic interaction mediated by Na^+ and K^+ ions. 4), DNA with proteins or peptides. 5), DNA dendrimers with marginal degree of polydispersity.

The biological approaches are mainly based on the viral carrier system. This system is relied on recombinant viral vectors either from weakened viruses such as poliovirus, vaccinia, hepatitis B virus and measles virus or from other viruses such asadeno-associated virus (AAV), alphavirus, human adenovirus (HAdV), vesicular stomatitis virus (VSV) and poxviruses. These virally vectored vaccines can induce effective specific immune responses against both expressed antigens and the viral capsid. They are generally conducted as the second vaccination after DNA priming in a prime-boost strategy (Cui Z *et al.*, 2003; Merdan T *et al.*, 2002; Otten G *et al.*, 2004; Mumper RJ, Cui Z, 2003; Bessis N *et al.*, 2004).

A more promising approach is electroporation (EP). This device produces electrical stimulation of muscle by a pulse generator and can be applied immediately after i.m injection of the DNA plasmid (Aihara H, 1998; Mir LM, 1999; Mathiesen I, 1999). This procedure enhances antigen expression by enhancing efficiency of transfection which can lead to local tissue injury and inflammation (Ahlen G, 2007) and consequently result in remarkably enhanced humoral and cellular immune responses (Babiuk S, 2002; Tollefsen S, 2002; Widera G, 2000; Marc D *et al.*, 2000). Importantly, EP reverses the failure of low-volume i.m injection on immune responses induction in mice and increases responses in larger animals. A combination of DNA priming with EP boost could induce higher antibody responses in comparison with responses that are induced by protein in complete Freund adjuvant, and also it can produce CTL responses. However, besides the transfection efficiency, an effective candidate may be the most critical factor affecting the DNA vaccine development.

1.3.5 Enhancement of efficiency of DNA Vaccines

Vaccine immunogenicity can be managed by factors which can attract APCs, enhance the uptake of plasmid DNA or provide additional co-stimulation. In these ways, immune responses can be directed toward a Th1 or Th2 response via different cytokine expression and distinct T cell activations. The injection of DNA vaccines with adjuvants encoding

cytokine can modulate the differentiation and expansion of Th1 and Th2 cytokines. For instance, Kim JJ and Sin JI (Sin JI *et al.*, 1999b;Kim JJ *et al.*, 1998a; Sin JI *et al.*, 1999c) described that the co-administration of IL12 encoded plasmid can direct the immune response toward a Th1. Also the HIV-1 vaccine co-administration with plasmids encoding IL12, IL18 and IFNγ could augment the level of antigen-specific proliferation in mice and rhesus macaques (Kim JJ *et al.*, 1999a). Kim JJ *et al* (1999b) showed that elevated levels of antigen-specific antibodies were correlated to IL4, IL10 co-administration and the chemokine macrophage-inflammatory protein-1α (MIP1α, Boyer JD *et al*., 1999) within an HIV-1 vaccine. In addition, treatment of TNFα could significantly enhance CTL responses and antigen-specific Th cell proliferation (Kim JJ *et al.*, 1998b). Table 1.7 provides some examples of enhancement of different immune responses by cytokines and other molecules (Chow YH *et al.*, 1998; Pan CH *et al.*, 1999;Babiuk LA *et al.*, 2003; Dale CJ *et al.*, 2004; Garmory HS *et al.*, 2005; Gurunathan S *et al.*, 2000; Liu MA *et al.*, 2006; Sumida SM *et al.*, 2004; Munks MW *et al*., 2004).

Table 1.7 Cytokines or adjuvants help to enhance certain immune responses

Intended response	Cytokines/ adjuvants
APC activation	CpG, CD40L, MIP1α, Flt3L
CTL response	IL1, IL2, IL12, IL15, IL18,GM-CSF, IFNγ, CD40L, ICAM1, LFA3
IgG2a (mice)	IL1, IL2, IL7, IL12, GM-CSF, IFNγ, CD40L
IgG1 (mice)	IL4, IL7, IL10, TGFβ
IFNγ-cellular response	IL1, IL2, IL7, IL12, GM-CSF, IFNγ, CD40L, ICAM1, LFA3
Protect activated T cells from death	CD137, CD134

Another way to enhance immune responses to DNA vaccines is associated with the addition of heterologous gene sequences encoding localization or secretory signals. Thus the cellular localization of heterologous antigen might be essential to manage the immune response. For example, stimulation with antigen secreted instead of localized on the cell membrane or within the cell induced higher titers of antigen-specific antibody in mice (Gurunathan S *et al.*,

2000; Locher CP *et al.*, 2002). Additionally, accelerated cytoplasmic antigen degradation and improved efficiency of MHC I antigen presentation were induced when ubiquitin sequence was fused with the antigen-encoding gen (Garmory HS *et al.*, 2005). CTL and B cell responses against the viral antigen were increased when antigen was fused with the heat-shock protein (hsp)70-binding viral J-domain (Reimann J and Schirmbeck R, 2004). While MHC class II responses were enhanced by fusing the antigen with the endosomal / lysosomal sorting signal sequence (derived from lysosome--associated membrane protein type 1; LAMP-1) (Kim TW *et al.*, 2003).

An alternative approach to augment DNA vaccine efficiency involves immunization with various combinations of recombinant virus, DNA vaccines and/or protein in *prime-boost* strategy. There are two types of *prime-boost* strategies, homologous and heterologous *prime-boost*. Among homologous *prime-boost* approaches, the DNA-prime-protein-boost approach uses recombinant protein antigens used in DNA prime immunization (Wang S *et al.*, 2008;Wang R *et al.*, 2004; Lu Y *et al.*, 2009).

Another DNA-prime-viral vector-boost approach focuses on the induction of T-cell immune responses. More immunogenic and protective effects than the DNA vaccine alone have been shown by DNA-priming and recombinant vaccinia boosting strategy in a mouse malaria model (Sedegah M *et al.*, 1998). Furthermore, complete protection in the same model has been obtained by boosting with the recombinant modified vaccinia virus Ankara (De´gano P *et al.*, 1999; Schneider J *et al.*, 1999). A similar manner is being exploited on viral vectors developed from fowpox (Kelleher AD *et al.*, 2006) and recombinant vesicular stomatitis virus (Egan MA *et al.*, 2005).

The heterologous '*prime-boost*" vaccination approach stimulates stronger expansion of antigen specific T cells by using different priming and boosting vectors (Schneider J *et al.*, 1999; Walczak M *et al.*, 2010). Compared to homologous *prime-boost* approach with the

same DNA vaccine, boosting a primary response with a heterologous vector could induce 4-10-fold higher T cell responses (Estcourt MJ *et al.*, 2002; Degano P *et al.*, 1999).

1.3.6 Possibility of DNA vaccine research on filariae

The most important factors that may determine the feasibility of development of a DNA vaccine against filariasis will be the identification of excellent parasite antigens and a clear understanding of the mechanisms of protective immunity. In the filarial life cycle, the L3 is a vulnerable stage of the parasite in the mammalian host in light of its sizes, numbers and migratory pathways. Several researchers have tried to address development of protective immunity against L3 stage both in experimental and human filariasis (Day KP *et al.*, 1991b; Devaney E, Osborne J, 2000; Eisenbeiss WF *et al.*, 1994; Bleiss W *et al.*, 2002; Helmy H *et al.*, 2000; Rajan TV *et al.*, 2002). Antibodies against the surface of L3 in human have been shown to be important to anti-larval immunity (Day KP *et al.*, 1991b; Rajan TV *et al.*, 2002). Protective immunity against larvae in host impacted by antibody-dependent cytotoxicity involving macrophages and eosinophils has been observed in experimental animals after vaccination with iL3s (Devaney E, Osborne J, 2000; Bleiss W *et al.*, 2002; Eisenbeiss WF *et al.*, 1994; Rajan TV *et al.*, 2002). The observation that IgM and IgG2 are the major L3 surface-reactive antibodies in human sera indicates the possibility that carbohydrate determinants may play a role in anti-larvae immunity. This is supported by the observation that significantly higher IgG2 antibodies to parasite carbohydrates are found in infection-free patients in endemic regions compared to infected humans (Mohanty MC *et al.*, 2001). Furthermore, gerbils are deficient in antibodies to T-independent carbohydrates antigens (Mohanty MC, Ravindran B, 2002). All the above indicate that the surface constituents of L3 may be crucial in protective immunity.

However, targeting stages other than L3 should not be discounted in the understanding of antifilarial immunity because distinct reactivities against adult male worm antigen have also been identified in putative immune humans (Turaga PS *et al.*, 2000). Importantly, adult

worms may be a main source of immuno-modulatory molecules (Harnett W, Harnett MM, 2006). The clearance of circulating Mf in infected animals were positively associated with circulating antibodies (Ravindran B *et al*., 2000), however an inverse relationship was also observed in both brugian and bancroftian filariasis (Ravindran B *et al.*, 1990; McGreevy PB *et al*., 1980). Moreover, as stated in the section 1.2, pathology in onchocerciasis is related to the death of Mf , which is mediated by Th2 immune responses. Thus the possibility of conflict between stimulating protection and exacerbation of disease must be taken into consideration. Additionally, control of Mf might be indirectly associated with the anti-fecundity instead of the antibody. Therefore, the focus should be put on anti-larvae and -adult instead of -Mf immunity

1.4 Vaccine candidates of filarial nematodes

Identification of vaccine candidates plays a key role in development of vaccines. Strategies to identify vaccine antigens in filariasis relied on serum antibodies to define antigens with comparison uninfected subjects with infected patients or by using sera from animals vaccinated with iL3s, or by molecular biological approaches to analyze and define new genes. Various candidates have been studied including the Abundant Larval Transcript (ALT), Venom Allergen Homologue (VAH), Cysteine Proteases and their Inhibitors (CPI), and Thioredoxin Peroxidase (TPX) (Table 1. 8).

1.4.1 Abundant Larval Transcript (ALT)

The ALT gene was first described from *B. malayi* (Gregory WF *et al.*, 1997) and it accounts for up to 5% of total transcript at the time when L3s invade the hosts. ALT is associated with two highly expressed genes, ALT-1 and ALT-2 (79% identity), which are homologous to an abundant gene from L3 of the *D. immitis* (Di-20/22L, Frank GR *et al.*, 1995), *O. volvulus* (Ov-ALT-1, Joseph GT *et al.*, 1998) and *A.viteae* (Pogonka T *et al.*, 1999). The secreted larval acidic protein (SLAP) produced by *O. volvulus* larvae (Bianco AE *et al*., 1990; Bianco AE *et al*., 1995) has also been shown to be a member of the ALT family (Gregory WF *et al.*,

2000) and is an orthologous to ALT in brugia (Wu Y *et al*, 2004).

These ALT proteins are considered to be attractive vaccine antigens for three reasons: 1) they are L3 specific and abundant but also expressed in L2 (Wu Y *et al*, 2004); 2) they are abundantly expressed and secreted; and 3) they have no homologues in the mammalian host. Gregory WF and colleagues (Gregory WF *et al.*, 2000) found a high frequency of IgG1 and IgG3 antibodies to ALT-1 and -2 in *B. malayi*- infected human and the response was different from the response to adult antigens which are targeted by IgG4. Ramachandran S *et al* (2004) found that 72% endemic normal individuals possessed antibodies against recombinant ALT-2 in endemic areas. This observation may suggest that the protective immunity might be associated with a response to this protein. When ALT-2 was tested in a jird model using either recombinant protein or DNA vaccine, significant protection (75% for protein and 57% for DNA vaccine, respectively, Thirugnanam S, 2007) was observed when compared to a control or adjuvant alone. This protection also indicates that this vaccine is a promising candidate for further development.

However, experiments also suggest that ALT proteins can modulate host immunity. Gomez-Escobar N *et al* (2005) described a transfection strategy to express these products in *Leishmania mexicana* by *in vitro* in macrophages and *in vivo* infection in mice. Results showed expression of ALT proteins instead of a truncated mutant led to 3-fold higher parasite burden *in vitro*, and ALT-transfected parasites caused severe disease *in vivo* and fewer mice eliminated infection. Furthermore, ALT-transfected parasites showed more resistance to worm killing induced by macrophages.

1.4.2 Cysteine Proteases and their Inhibitors (CPI)

CPIs or cystatins were first identified from *O. volvulus* L3s and adult stages (Lustigman S *et al*, 1991) then were found in *B. malayi* L3s (Gregory WF *et al*., 1997). In these parasites three forms have been identified, Bm-CPI-1 is both secreted and located on the surface of L3

although expression stopped after just 2 days post infection in mammalian hosts. Bm-CPI-1 may be a target for antibody-mediated immune responses because of the L3 surface location. Bm-CPI-3 has also been identified and is expressed by L3s. Both of them were studied less than Bm-CPI-2, which is expressed throughout the full life cycle. Bm-CPI-2 has been shown to inhibit antigen presentation through MHC II (Manoury B *et al.*, 2001; Murray J *et al.*, 2005) and may modulate the host's immune response to the parasite. Bm-CPI-2 has been found bi-functional by comparing with vertebrate cystatins (Murray J *et al.*, 2005) with one to block papain-like proteases and one to suppress a legumain such as asparaginyl endopeptidase (AEP). Site-directed mutagenesis on Bm-CPI-2 at Asn-77 (the residue associated with AEP inhibition) to Asp and Lys showed 10-fold decreased activity in AEP inhibition, respectively, whereas blocking of papain-like proteases showed only a small degree reduction.

Cystatin homologues have been found in other filariae such as onchocystatin (including Ov-CPI-1, Ov-CPI-2) from *O. volvulus* (Lustigman S *et al.*, 1996). They have been shown to modulate T cell and macrophage activities and thus contribute to the hypo-responsiveness in onchocerciasis patients (Schönemeyer A, *et al.*, 2001), moreover, it has been shown partial protection in its unmodified form with alum adjuvant (Abraham D *et al*, 2001). A cystatin from *A. vite*ae (Av-CPI) was reported to be highly secreted by female adult worms. its recombinant protein is able to inhibit proliferative responses and increase IL10 production, again indicating an immuno-modulatory function (Hartmann S *et al.*, 1997).

An immuno-modulatory role for cystatins is also suggested by works on *L. sigmodontis*. The level of nitric oxide (NO) was reduced while TNFα was increased when C57BL/6 mice were simultaneously inoculated with *L. sigmodontis* Mf and given recombinant Ls-CPI (Ls cystatin) protein via micro-osmotic pumps (Pfaff AW *et al.*, 2002). In addition, antigen-specific proliferative responses of spleen cells to Mf were decreased, despite antibody production was not inhibited. Produce protective immunity was not obtained by vaccination of BALB/c mice with

Ls-CPI but the number of vaccinated mice that became microfilariamic (potent) was reduced (Pfaff AW *et al.*, 2002).

1.4.3 Venom Allergen Homologue (VAH)

VAH family of proteins or *Ancylostoma* secreted protein (ASP) produced by infective L3 are cysteine rich secretory proteins (CRISP). Several homologues of CRISP have been reported from nematodes such as *A. caninum* (Hawdon JM *et al.*, 1996), *Haemonchus contortus* (Rehman A, Jasmer DP, 1998), *Ancylostoma duodenale* (Bin Z *et al.*, 1999), *C. elegans*, *Ascaris*, *N.americanus* (Daub J, 2000), *O. volvulus* (MacDonald AJ *et al.*, 2004), *Toxocara canis* (Tetteh KK, 1999), and *Strongyloides* (Hawdon JM *et al.*, 1999). Some experiments have suggested that VAH is a potential vaccine candidate. For example, 80% protection was obtained by vaccination with Hc-24 after challenge with *H. contortus* in sheep (Schallig HD, van Leeuwen MA, 1997), and ASP-1-immunized mice showed up to 80% reduction of worm burden with comparison to controls (Ghosh K *et al.*, 1996; Sen L *et al*., 2000).

Murray J *et al* (2001) observed that more than 95% of microfilaraemic subjects are antibodies positive against Bm-VAL-1 with high levels of IgG3 and IgG4. Anand SB *et al* (2007) suggested EN individuals produced *W. bancrofti* VAH (Wb-VAH)-specific IgG1, IgG2 and IgG3 whereas patients with chronic pathology mainly generated IgG3.

Ancylostoma secreted proteins (ASP) have also been well documented on protective immune responses induction against hookworm infections in mice, hamsters and humans (Sen L *et al.*, 2000; Goud GN *et al.*,2004; Bethonya JM *et al.*,2008)

1.4.4 Thioredoxin Peroxidase (TPX)

TPX is a member of the thiol-specific antioxidant (TSA) family which has been described from prokaryotes and eukaryotes. This family was originally described from yeast (Kim IH , 1989; Kim K *et al.*, 1988) as a 27 kDa ‘protector’ protein which was able to protect other

proteins from oxidative damage. This protein was also able to reduce H_2O_2 and alkyl hydroperoxides (Chae H *et al.*, 1994). The TPX gene (s) has been found in *O. volvulus* and *B. malayi*. In *O. volvulus*, the Ov-TPX-2 cDNA represents about 2.5% of the total cDNAs from the L3 cDNA library (Wenhong Lu *et al.*, 1998) and encodes a 22 kDa native protein found in both L3 and adult stages. In larvae, Ov-TPX-2 protein is mainly localized to the hypodermis and cuticle. In adult worms, the primary sites are the uterine epithelium and intestine (Lu W *et al.*, 1998). The *B. malayi* Bm-TPX-1, locates in the cells of the hypodermis/ lateral chord in adults, was only 60% identical to putative TPX proteins from *O. volvulus* and *C. elegans* and is also different to Bm-TPX-2. The distribution of this protein in the parasite suggests that Bm-TPX-1 may play an important role in countering radicals produced within cells (Ghosh I *et al.*, 1998).

TPX is also found in *Fasciola hepatica*, in which it appears to induce recruitment and alternative activation of macrophages (Donnelly S *et al.*, 2005) and it is also found in schistosome eggs (Williams DL *et al.*, 2001). The native TPX-1 protein elicited significant greater proliferation and up-regulation of IFNγ, IL2, IL4 and IL5 in $CD4^+$ cells from infected CBA and C57BL/6 mice. By comparison, recombinant TPX-1 elicited a smaller Th1-biased response, with significant production of IFNγ and IL2 (Williams DL *et al.*, 2001). A comparative study of the physiological roles of three peroxidases (NADH peroxidase, alkyl hydroperoxide reductase and thiol peroxidase) in oxidative stress responses suggested that TPX is the most important antioxidant for protecting the cells from the phagocyte environment (La Carbona S *et al.*, 2007).

Table 1. 8. Vaccine candidates of filariae

Gene name	Expressed by	Antigen type	protection	Function	Refs
ALT	L3	secretory	70% (*B. malayi*)	Vital in transmission and infectively of filariae	Gregory. WF *et al*., 2000
CPI2	L3/adult	Secretory/ surface		Inhibits class II MHC-restricted antigen processing; Vaccine-mediated protection.	Murray J *et al.*, 2005; Abraham D *et al*, 2001)
VAH	L3/adult	secretory	64% (*B. malayi*)	Induce angiogenesis; potent adjuvant	Murray J *et al.*, 2001
TPX	All stages	cuticular		Avoid oxygen radical-mediated damage	Ghosh I *et al.*, 1998; Lu W *et al*., 1998

1.5 The *L. sigmodontis*/ mice research model

A major constraint of filarial diseases research and vaccine development has been the lack of great animal models in which the parasite can go through full life cycles. Early animal models include *O. volvulus* in mice, *A. viteae* in gerbils and *B. malayi* in mice and gerbils, but all these models have limitations. For example, although *O. volvulus* can survive in mice after surgical implantation, it cannot produce Mf in the pleural cavity (Lustigman S *et al.*, 2003). Similarly, *Brugia* species are found to have an incomplete life cycle in mice. Although *B. malayi* and *A. viteae* can reach potent stage with Mf in mongolian gerbils (*Meriones unguiculatus*), lack of research reagents limit the immunological investigations (Lawrence R, Devaney E, 2001; Abraham D *et al.*, 2002).

Major progresses have been made in two animal models. *O. ochengi* in cattle is a good model for onchocerciasis research based on the merits that it is the closest known relative of *O. volvulus* and it is also transmitted by the blackfly, *Simulium damnosum* (Trees AJ *et al.*, 2000). Nodule formation caused by *O. ochengi* resembles that caused by *O. volvulus* and is

convenient for immunological analysis. However, it does not cause evident pathology in cattle (Allen JE *et al*., 2008).

Another excellent model is the *L. sigmodontis*- lab mouse model. The report by Petit G *et al* (1992) described that *L. sigmodontis* was able to produce potent infections in BALB/c mice. This was a major breakthrough in filarial research because this raises a possibility to carry out detailed immunological researches for the first time. *L. sigmodontis* development in different mouse strains is listed in table 1.9. However, not all BALB/c mice infected with *L. sigmodontis* can develop potent infections. The one constraint of the *L. sigmodontis* mouse model is that there is little or no pathology associated with the infection nor the clinical signs which are normally associated with onchocerciasis or lymphatic filariasis. This, in part, can be attributed to the different anatomical compartments occupied by adult worms (thoracic cavity) and Mf (blood) (Table. 1.10).

Table 1.9 Maturation of *L sigmodontis* in different mouse strains (from Petit G *et al* (1992))

Mouse strains	Haplotypes	Susceptible	Resistant	Patency
BALB/c	H-2d	yes	no	yes
BALB/K	H-2k et	Less than BALB/c	yes	no
BALB/B	H-2b	Less than BALB/c	yes	no
B10	H-2b	no	yes	no
B10Br	H-2k et	no	yes	no
B10D2	H-2d	no	yes	no
CBA/Ca	H-2k et	Adult found		no
CBA/HN	H-2b	Adult found		no
C3H/HeN	H-2k	Adult found		no
DBA/2N	H-2d	Adult found		no

Note: Male CBA/HN and C3H/HeN were more susceptible to infection than female mice. Inverse phenomenon was observed with strains BALB/c; and, no host sex effect was seen in DBA/D2N.

The life cycle of *L. sigmodontis* in the mouse is summarized in Fig 1.5. Females give birth to Mf 8 weeks after infection, then the Mf migrate to the blood from where they can be ingested by blood-feeding arthropods. In the laboratory the mite, *Ornithonyssus bacoti*, is

used as a vector. Ingested Mf then moult to L2 5-6 days post ingestion and to L3 8 days post ingestion. L3s become infective to the vertebrate host 1 day after the L2/L3 moult (Diagne *et al.*, 1989). Infective L3s are inoculated to the vertebrate host when mites feed. The L4 moults 7-8 days post infection when the parasites are located in the pleural cavity. The final moult to adult occurs 28-35 days post infection. Mf can be detected in circulation 55-60 days post infection.

Table 1. 10. General features of the biology of *O. volvulus*, *O. ochengi*, and *L. sigmodontis* (cited from Allen JE *et al.*, 2008)

Filariae	Vector	Time to patency	Adult	Mf	Constraints	Merits
O. volvulus	*Simulium spp*.	250-375 days	Subcutaneous nodules	Skin	Experiment not possible	The target organism
O. ochengi	*Simulium spp*.	More than 250 days	Intradermal nodules	Skin	No pathology	Closely related to *O. volvulus.* Quantifiable test under natural challenge.
L. sigmodontis	*O. bacoti*	Up to 50 days	Pleural cavity	Blood	No evident pathology	Full life cycles accessible for test. Murine immunology accessible. Protective immunity elicited by vaccination

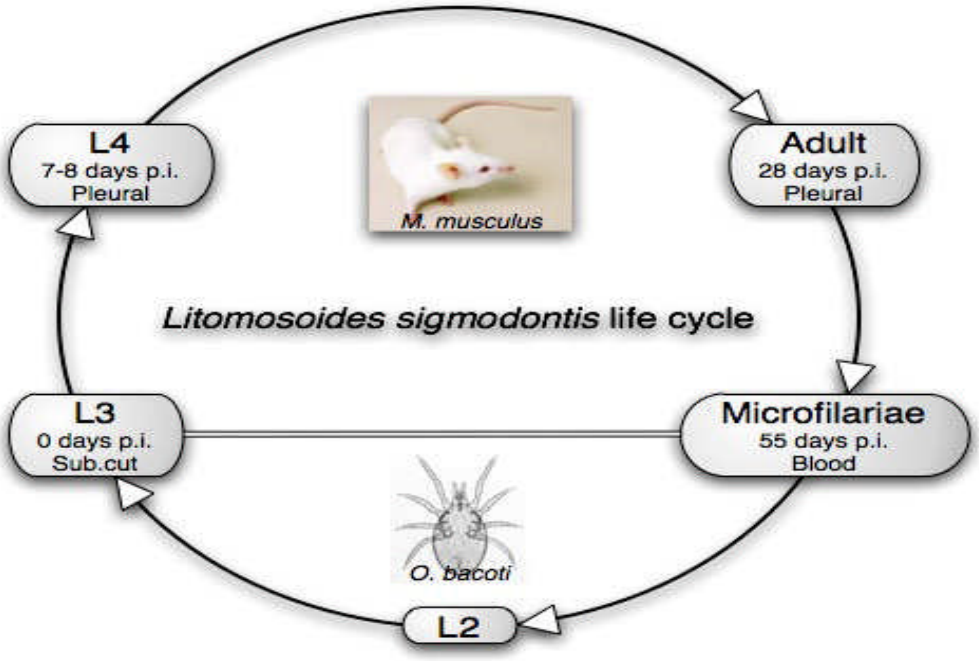

Fig. 1.5 Life cycle of *L. sigmodontis* (courtesy of Simon Babayan)**.** *O. bacoti* mite is used to maintain the life cycle of *L. sigmodontis* during L1 to L3. BALB/c mouse is infected with infective L3 and can reach patency in 55-60 days after infection.

1.6 Designing a DNA vaccine against filariae

To design an effective vaccine against filarial nematodes, the necessity to know the history of such vaccine development is required. Since 1950s, a vaccine against *D. viviparous* consisting of iL3s had been applied successfully to control bronchitis. Such success excited scientists to develop vaccines against filarial nematodes in terms of the similar principle and procedure. Many irradiated vaccines had been examed in the past decades and showed quite high protections (from 70% to 100%, Wong MM *et al*, 1974*;* Abraham D *et al*, 2002; Grieve RB *et al*, 1998). Even the protective immunity induced by iL3 vaccination was found to be Th2 bias (Taylor MJ *et al*., 1994; Johnson EH *et al.*, 1998; Lange AM *et al*., 1994), which was essential to attack the filarial nematodes (see 1.2). However, the iL3 vaccine has its weakness: it could never be used on humans because of safety concerns and the limited acceptance mentally. Also, iL3 vaccine was unstable, it was not feasible for global distribution. Moreover, this vaccine must be given annually which is labour-intensive.

A more reasonable approach for vaccination would be the use of recombinant antigens. The first step to develop a recombinant vaccine is the identification of candidates. On *B. malayi*, Maizels RM *et al* (2001) developed three approaches to identify immune evasion genes which might be the candidates for a novel vaccine. First strategy aimed to characterize surface or secreted antigens, in which Bm-CPI2, a member of the cystain, had been digged out. It had been tested as a recombinant vaccine and showed a promise effect (see 1.4), in parallel, a serpin, Bm-SPN2, had also been found. The second route they used was based on the hypotheses that filarial nematodes might encode homologues of cytokines from mammalian hosts. They found that the Bm-TGH2, which is a homologue of TGFβ, could bind to TGFβ receptors. Likewise, the Bm-MIF had been shown high similarity to the proteins from mammalian host in both structure and function. The third method was to select abundant mRNA from the key points of its life cycle. By this way, Bm-ALT and Bm-VAL-l had been hunted. These candidates had been examined by their recombinant forms in animal models and showed different protective immunity-inducing features (see 1.2, 1.4). In the case of *O. volvulus*, a program to develop a recombinant vaccine was the Edna McConnell Clark Foundation`s (EMCF) Oncho Task Force`s network (Cook JA *et al*., 2001). They tested 44 recombinant antigens in 12 laboratories. Among them, 14 antigens had the capabilities to induce partial but significant protections with the adjuvants of alum or FCA (Lustigman S *et al*, 2002). Further, Ov7, Ov64 and OvB8 recombinant proteins were individually tested in mice for their protective features. Significant reductions of parasite survival were obtained with adjuvant alum instead of FCA, suggesting the protective immunity induced would be Th2 instead of Th1 responses (Kenney JS *et al*, 1989; Yip HC *et al*, 1999; Forsthuber T *et al*, 1996). However, fructose-1, 6-biphosphate aldolase functioned with the addition of FCA not alum (McCarthy JS *et al*, 2002). A more recent field trial vaccination in cattle- *O. chengi* system in West Africa was performed by using 8 recombinant antigens which were expressed in *O. chengi*. These antigens were administrated with either FCA or alum individually, results showed 58% reduction of parasite infections (Makepeace BL *et al.*, 2009). Although the recombinant vaccines have been success in some

laboratory animal models and field trials, the expression of nematode proteins in prokaryote bacterial *E. coli which* has different bio-machine to eukaryote, may lead to the production of proteins with abnormal secondary or tertiary structures or lack of post-translational modifications. Consequently, their absence of bio-activities may impair their functions in host cells. Despite new expression systems such as yeast, insect cell and mammal expression systems, which improve the activity of post-translational modifications greatly, have been applied extensively on protein expressions, the expressed proteins in these systems cannot act as native proteins or proteins expressed in host cells (Y Gao, 2000). Moreover, as stated above, some identified antigens induce Th1 whereas others stimulate a Th2 response, the direction of protective immunity cannot be managed when recombinant proteins are used. Therefore, an alternative strategy is needed to mimic the function of native protein and to direct the protective immunity that the administrated vaccine is designed to induce.

The DNA vaccine is a novel type of vaccine which has many advantages over the traditional vaccine as stated in section 1.3. The point that DNA vaccine can express carried gene by using the bio-machine system in host cells naturally and the expressed protein is more close to its native form in secondary or tertiary structure is noteworthy. Because this is what the recombinant protein produced in *E. coli* or other protein expression system cannot provide. In addition, the immunity the vaccine requires to induce can be managed by encoding different cytokine adjuvants (see 1.3.5), this fortune makes DNA vaccine more feasible and easy managed. As a support, DNA vaccine had been shown promise on controlling infective disease and even two commercial products had been allowed to distribute (see 1.3). However, till now, most experiments were performed on laboratory experimental animals instead of primates or even big animals. The major reason was associated with the low efficiency that the DNA vaccine induced. However, this weakness has been overcome by the use of electroporation (see 1.3.4), which significantly improves the DNA efficiency in big animals and reduces the amount of DNA.

However, to design a DNA vaccine must understand the details of protective immunity that a vaccine should induce. It is generally accepted that infection with helminths is associated with Th2 responses characterized by increased IL4, IL5 and IL13. This can be seen in both natural and experimental infections with platyhelminths (including schistosomes) and nematodes (including filarial parasites) (see 1.2). However, the Th1 responses cannot be ignored (see 1.2). Moreover, evident conflicting data on the role of Th1 versus Th2 responses in protective immunity in lymphatic filariasis and onchocerciasis may be a manifestation of the complexity of the filarial life cycle (see 1.2). This arouses more recognition of Tregs. Apparently, the balance of Th1/ Th2 highlights the need for a thorough understanding of the mechanism of protective immunity to ensure development of an effective vaccine (Fig 1.6).

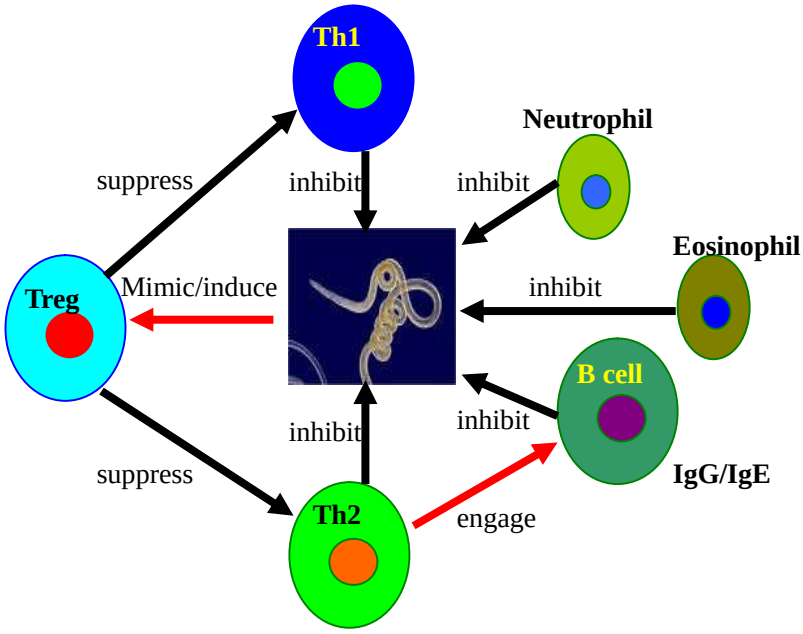

Fig 1.6 Network of immune responses associated with worm killing. Th2 cytokines including IL4, IL5, IL13 are associated with worm and Mf killing. Th2 cytokines may engage the B cells to produce antibodies including IgG1, IgG2a, IgE to inhibit the worm and Mf development. Eosinophils and neutrophils are also involved in worm expulsion by degranuation or encapsulation around the worms. Worms can trigger Tregs to downregulate Th1, Th2 and other effectors. Treg also can regulate response to avoid severe pathology.

1.7 Aim of PhD

A long term objective is the development of a vaccine against onchocerciasis and lymphatic filariasis. The aim of the present work in this thesis is to provide proof of principle for use of a DNA vaccine against filariasis. Considering filarial infections persist long life time in host, a hypothesis was proposed that this may due to the filariae-triggered Treg responses and the existence of parasite-derived immuno-modulations. To develop an effective DNA vaccine regimen against filariasis that can circumvent immune regulation and immuno-modulation, *L. sigmodontis*/mice model will be used to test the selected filarial antigens including Acidic Domain Deleted Abundant Larval Transcript (ADDALT), Mutated Cysteine Proteases and their Inhibitors (CPImu), Venom Allergen Homologue (VAH), Thioredoxin Peroxidase (TPX). Also electroporation will be used to enhance the efficiency of DNA vaccines following the injection of DNA plasmids. Cytokines/ chemokines macrophage inflammatory protein 1 alpha (MIP1α), fms-like tyrosine kinase 3-ligand (Flt3L), and interleukin 4 (IL4) will be used as adjuvants. All these are intended to test selected antigens for their abilities to evoke protective immune responses.

Chapter 2-Materials and Methods

2.1 Materials

2.1.1 Mice

BALB/c mice were used for all experiments. These animals were either bred in house in the Ann Walker Facility, University of Edinburgh or purchased from Harlan-UK (Bicester, UK). All mice were between 6 and 8 weeks old at the beginning of the experiment and were euthanized with Vetelar/ Domitor before necropsy. They were housed in individually ventilated cages (IVC) and were given sterilized food and water. All procedures conformed to the Animals (Scientific Procedures) Act 1986, United Kingdom

2.1.2 Parasite life cycle maintenance

The filarial parasite *L. sigmodontis* was maintained in house by Alison Fulton. 1-3 C57BL/6 or BALB/c baby mice were put into each colony flask containing mites (*O. bacoti*). Within 18 hours the baby mice were removed from the colony flasks and 450 female mites per flask were collected and put into a new colony flask for 1 week's incubation at 37°C and 70% humidity. After one week, the flasks with non-infected mites were put on infected jirds (*M. unguiculatus)* carrying Mf in the blood stream and kept at 37°C and 70% humidity overnight. The infected mites from infected jirds were collected into autoclaved tubes (50 mites per tube) and incubated at 37°C and 70% humidity for 12 days to obtain the infective larvae. Infective larvae were collected by dissecting the mites, then the L3s were put in RPMI 1640 medium for inoculation in room temperature after counting and were ready for experimental use.

2.1.3 Parasite antigens preparation

For parasite antigen preparation, adult *L. sigmodontis* worms were collected from BALB/c

mice (see 2.1.2) and homogenization (without protease inhibitors) of mixed sex worms using a ground-glass homogeniser in PBS on ice was carried out. The homogenate was centrifuged at 10,000×g for 20 minutes and the resultant supernatant fluid was passed through a 0.22 μm filter before determination of protein concentration using the Bradford protein assay. The assays were read at 595 nm and concentration of antigen preparations were determined by reference to the standard curve of 2-fold dilutions of bovine serum albumen (BSA) at a starting concentration of 2mg/ml. The antigen preparations were stored at -20°C.

2.1.4 Buffers, solutions and media

Carbonate buffer for ELISA coating: two solutions were prepared: A, 8.5g $NaHCO_3$ in 1 liter distilled water; and B, 10.6g Na_2CO_3 in 1 liter distilled water. Then add 45.3 ml A plus 18.2 ml B, make up to 1 liter with distilled water, and adjust the pH to 9.6.

Luria-Bertani broth (LB) for bacterial culture: 10 g tryptone, 5 g yeast extract, 5 g NaCl, distilled water to 1 liter, sterilized by autoclaving (121°C, 1.5 pounds per square inch (psi), for 30 minutes)

LB-agar: 1.5% Difco agar in LB, sterilized by autoclaving (121°C, 1.5 psi, for 30 minutes)

10×PBS: 8.0 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4, 2 g KH_2PO_4, distilled water to 1 liter, pH 7.5, sterilized by autoclaving (121°C, 1.5 psi, for 30 minutes).

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10×TBE: 121.0 g Tris base, 61.8 g boric acid, 18.6 g EDTA, distilled water to 1 liter, pH 8.0.

TBS: 8.75 g NaCl, 2.42 g Tris base, distilled water to 1 liter, pH adjusted to 7.4 with HCl.

50×TE: 60 g Tris base, 100 ml 0.5 M EDTA, distilled water to 1 liter, pH 8.0.

S.O.C broth: 20 g bacto tryptone, 5 g yeast extract, 10 ml 1M NaCl, 2.5 ml 1M KCl were mixed in 970 ml distilled water and sterilized by autoclaving (121°C, 1.5 psi, for 30 minutes), the broth was completed by addition of 10 ml 1M $MgCl_2$, 10 ml 1M $MgSO_4$, 10 ml 2M glucose (all these regents were filtered by passage through 0.22 μm filter).

1% or 2 % agarose gel: 1g or 2g agarose was added into 100 ml 0.5×TBE buffer and dissolved at 100°C for 15 minutes in microwave oven, with addition of 5μl 10 mg/ml ethidium bromide (EB) and the mixture was poured into the gel mould and allowed to set for 15-30 minutes before use.

SDS Gel: ready to use precast NuPAGE 4-12% Bis-Tris gels (1.0 mm×10 well, cat no. Np0321 Box) were purchased from Invitrogen.

Complete RPMI1640: RPMI1640 media was purchased from Gibco, UK (cat no 42401018) and before use the following were added into 500 ml: 2 mM glutamine; 10 mM 2-hydroxyethyl (Hepes), 10% fetal calf serum (FCS), 100 U/ml penicillin,100 μg streptomycin.

Protein purification buffer: 4 buffers were used to purify His-Tag recombinant proteins within AKTA system including Charge Buffer, Strip Buffer, Binding Buffer and Elution Buffer.

His-Tag protein affinity purification Charge Buffer Stock (8 ×): 400mM $NiSO_4$.
1×Charge Buffer: add 25 ml 8 × Charge Buffer Stock in 175 ml distilled water.

His-Tag protein affinity purification Strip Buffer Stock (8 ×): 400 mM EDTA, 2 M NaCl and 80 mM Tris HCl, pH7.9.
1× Strip Buffer: add 25 ml 8× Strip Buffer Stock into 175 ml distilled water.

His-Tag protein affinity purification Stock solutions for Binding Buffer Stock: 4 M NaCl: 234 g in 1 liter distilled water.

4 M Imidazole: 136.2 g in 500 ml distilled water.

0.2 M Na_2HPO4: 28.4 g in 1 liter distilled water.

0.2 M NaH_2PO_4: 13.6 g in 500 ml distilled water.

0.2 M Sodium Phosphate: 40.5 ml of 0.2 M Na_2HPO4 and 9.5 ml of 0.2 M NaH_2PO_4, pH7.4.

1× Binding Buffer: 100 ml of 0.2 M Sodium Phosphate, 125 ml of 4 M NaCl, 2.5 ml of 4 M Imidazole, made up to 1 liter distilled water before use.

His-Tag protein affinity purification 1×Elution Buffer: 20 ml of 0.2 M Sodium Phosphate, 25 ml of 4 M NaCl, 25 ml of 4 M imidazole, made up to 200 ml distilled water then added 6 M urea before use.

Western blotting Transfer buffer: NuPAGE Transfer Buffer (20×, cat no. NP0006-1) was supplied by Invitrogen and 20 fold diluted prior to use with distilled water.

SDS running buffer: NuPAGE MES SDS running buffer (20×, cat no. NP0002) was supplied by Invitrogen and 20 fold diluted prior to use with distilled water.

SDS Gel Stain solution: dissolve 0.4 g of Coomassie blue R350 in 200 ml of 4 % (v/v) methanol in water with stirring as needed. Filter the solution to remove any insoluble material. Add 200 ml of 20 % (v/v) acetic acid in water. The final concentration is 0.1 % (w/v) Coomassie blue R350, 20 % (v/v) methanol, and 10 % (v/v) acetic acid.

SDS Gel destain solution: add 500 ml of high performance liquid chromatograohy (HPLC)-grade methanol to 300 ml of water. Add 100 ml of acetic acid, after mixing, adjust the total volume to 1000 ml with water.

SDS Gel Storage solution: add 25 ml of acetic acid to 400 ml of water. After mixing, adjust the final volume to 500 ml with water.

2.1.5 Bacterial host strains and plasmid vectors

Bacterial strains and vectors used in experiments are listed in Appendix 1. All vectors were kept at -20°C and strains were kept at -80°C (see appendix 1).

2.1.6 Primers for DNA amplification and sequencing

All primers used for DNA amplification by PCR and DNA sequencing were synthesized by the Invitrogen company, and all primers are included in the tables (see Appendix 2.1, 2.2).

2.1.7 Kits

Kits were purchased for plasmid extraction, PCR product purification and gel extraction including QIAprep spin Miniprep kit (Cat no.2714), QIAGEN plasmid Maxi kit (Cat no. 12162), QIAquick PCR purification kit (Cat no. 28104) and QIA quick Gel Extraction kit (Cat no. 28704). All kits were obtained from QIAGEN, UK.

2.2 Methods

2.2.1 Methods of DNA analysis and manipulation

2.2.1.1 RNA extraction

For the isolation of total RNA, worms or tissue samples were removed from the gerbils or mice (see 2.1.2) and COS7 cells obtained from cell cultures were also used as source of mRNA for specific experiments (see 3.2.5 and 4.2.5). All samples were stored in 1 ml RNALater (Ambion) or Trizol (Invitrogen) at -80°C. Before processing, the samples were incubated for 5 minutes at room temperature, and once the sample was completely thawed, 150 µl chloroform per 800 µl sample was added, shaken vigorously by using a shaker for 15

seconds and incubated further for 2-3 minutes at room temperature, followed by centrifugation at 10,000 g for 20 minutes at 4°C. The aqueous phase was transferred to a new tube and 1 ml of isopropanol containing 0.5 µl glycogen was added and the mixture was vigorously shaken for 15 seconds. After incubation for 10 minutes at room temperature, the samples were centrifuged at 10,000 g for 20 minutes at 4°C. The supernatant fluid was removed and RNA pellet resuspended in 1 ml 75% ethanol. The RNA was recovered by centrifugation at 10000 g for 20 minutes at 4°C and after removal of the supernatant fluid, the RNA pellet was allowed to dry at room temperature. To the dried RNA pellet, 20µl dethyl pyrocarbonate (DEPC) water was added and the sample was incubated at 60°C for 10 minutes to dissolve. RNA was stored at -80°C until to use.

2.2.1.2 First strand cDNA synthesis

1 µl of RNA was used to synthesize the first strand cDNA. The total reaction volumes were 20 µl and composed of : 1 mM of dNTP (Promega), 0.5 µg oligo dT (Promega), 1U RNase inhibitor (Promega) and 500 U MMLV reverse transcriptase (Stratagene) in 1× reaction buffer (Stratagene). The reaction mixture was incubated at 20°C for 10 minutes followed by 37°C incubation for 1 hour and 99°C for 5 minutes. The cDNA was stored at -80°C.

2.2.1.3 Standard PCR reactions

Standard PCR reactions were performed in a reaction mixture containing: 1-2 µl cDNA, 0.5 µM each primer, 0.25 mM dNTP, 1× PCR reaction buffer (QIAGEN) and 2.5 U Taq polymerase (QIAGEN) in a total volume 20 or 50 µl (depended on how much products required). The reaction conditions were as follows: 94°C for 5 minutes or 10 minutes (if the templates were bacterial clones), then 30 cycles of 30-60 seconds at 94°C, 30-90 seconds at 55°C and 30-90 seconds at 72°C (depending on the size of the product to be attained), an additional 10 minutes at 72°C were required to extend target PCR products. All PCR products were examined by gel electrophoresis (0.8-1% agarose, in 0.5× TBE buffer, 120 V for 40-50 minutes) and visualized with 5µl ethidium bromide (EB) per 100 ml agarose

solution followed by image capture performed on the Gel Image system (Bio-Rad).

2.2.1.4 Real-time PCR

Relative quantification of genes of interest was detected by real-time PCR using a LightCycler machine (Roche Molecular Biochemicals). Initially, a positive control was determined by preliminary real-time PCR, identifying a reaction is considered to be a positive control with a good standard amplication curve. In each reaction the positive control sample of cDNA was 6 fold diluted from initial 1:10 dilution and was used as a standard curve to estimate the expression levels of genes. PCR amplifications were performed in 10 µl containing 3 µl cDNA, 1.4 µl SYBR Green I Master distilled H_2O (Promega), 0.3 µl forward and reverse primer each, and 5 µl SYBR Green I Master (Promega). The reaction was carried out under the following conditions: 30 seconds denaturation at 95°C, 5 seconds annealing of primers at 60°C and 20 seconds elongation at 72°C for 60 cycles. The amplication efficacy was measured and data were recorded automatically. .

2.2.1.5 DNA isolation and purification

DNA isolation and purification was carried out according to the manufacturer's instructions of the QIA quick Gel Extraction kit. Gel electrophoresis (in 0.5× TBE buffer, 120 V for 40-50 minutes) was performed to visualize the target gene fragment band, which was the excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a colorless tube, and Buffer QG was added in a ratio of 1 to 3 weight to volume. The gel was incubated at 50°C for 10 minutes. After the gel slice had dissolved completely, 1 gel volume of isopropanol was added to the sample and mixed. The sample was applied to a QIAquick 2ml spin column in a provided 2 ml collection tube. To bind DNA, apply the sample to the QIAquick column and centrifuged for 1 minute at 10,000 g. Unbound material was removed by addition of 0.75 ml of Buffer QG to the QIAquick column and centrifuged for 1 minute at 10,000 g. The QIAquick column was transferred into a clean 1.5 ml microcentrifuge tube and bound DNA eluted through addition of 50 µl of Buffer EB (10mM Tris-Cl, pH 8.5) and

centrifuged for 1 minute at 10,000 g. Purified DNA was stored at -20°C.

2.2.1.6 Plasmid construction

All plasmids used in experiments were constructed by using the “digestion-ligation” method (Fig 2.1) and are listed in Appendix 3. Recombinant plasmids of pDEC-OVA and its corresponding control pISO-OVA (Nchinda G *et al.*, 2008) were a kind gift from the Laboratory of Cellular Physiology and Immunology, the Rockefeller University. The constructions of pDEC-OVA and its control pISO-OVA are illustrated in Fig 2.2. The constructions of pSiEOS and pSiEOSSC are illustrated in Appendix 4, and constructions of pET vectors are illustrated in Appendix 5.

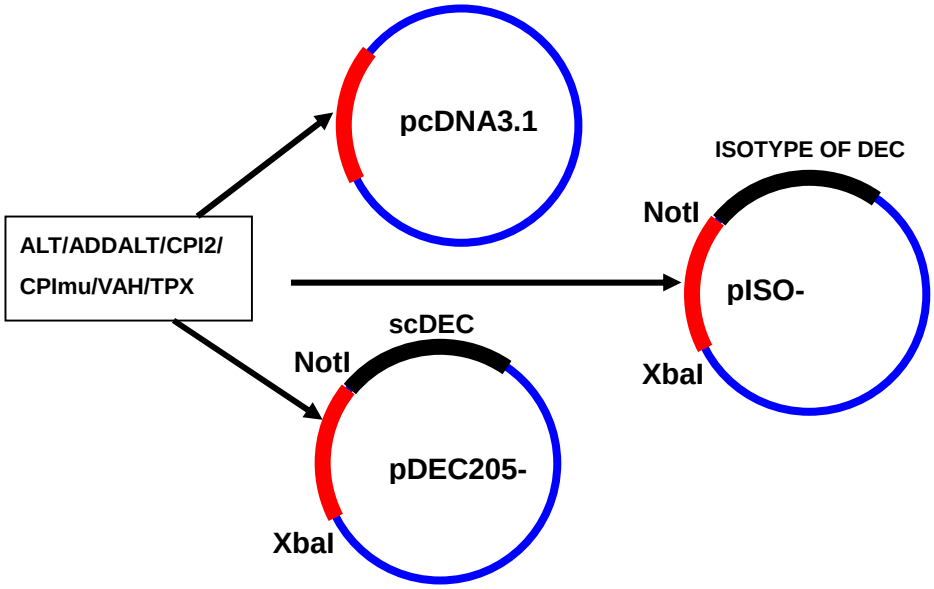


Fig 2.2 Schematic of construction of ALT, ADDALT, CPI2, CPImu, VAH and TPX plasmids.

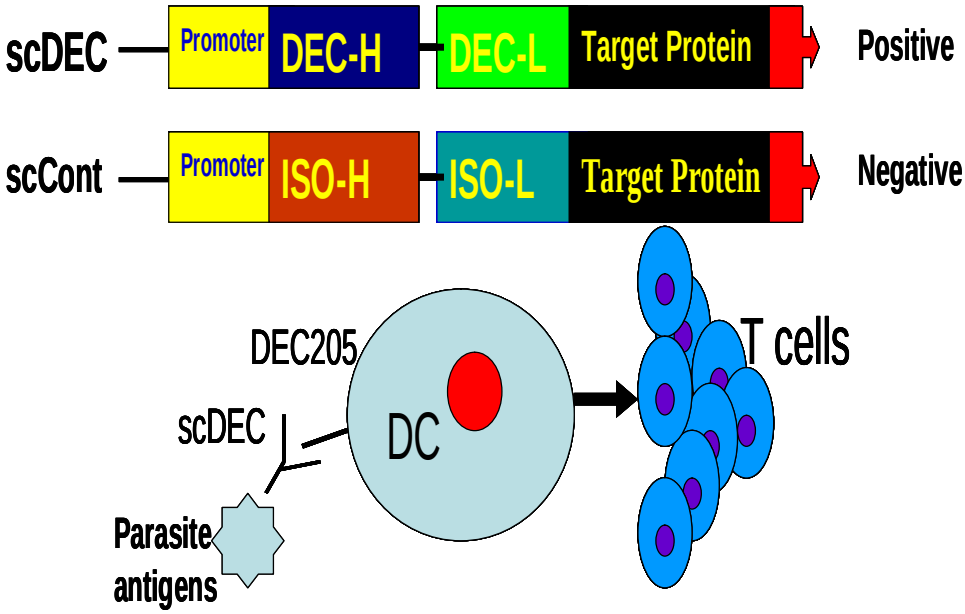


Fig 2.2 Construction strategy of pDEC-OVA and the control pISO-OVA, and interaction between parasite antigens and DCs via DEC205 receptor. The "Target protein" represents OVA gene or any parasite gene which is required for the DNA vaccine. Antibody scDEC represents single chain anti-DEC205 antibody, which can specifically interact with DEC205 receptor locate on the surface of DCs. The scDEC205 was constructed by fusion of heavy- and light-chain variable regions of monoclonal antibody NLDC145 to DEC205 into a pcDNA3.1 vector. The scCont antibody is the isotype of scDEC205 but cannot bind to DCs.

2.2.1.7 Gene site directed mutation

Mutation of the CPI2 gene was carried out according to the manual of the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Cat no.200519). Two complimentary oligonucleotides containing the desired mutation of asn66 to lys66 (Fig 2.3) flanked by unmodified nucleotide sequence were synthesized. Then the reaction was prepared as follows: 5 µl of 10× reaction buffer, 1µl (5-50 ng) of dsDNA of CPI2, 1 µl (125 ng) of forward primer , 1 µl (125 ng) of reverse primer, 1 µl of dNTP mix, and distilled water to a final volume of 50 µl, then 1 µl of PfuTurbo DNA polymerase (2.5 U/µl) was added. The conditions were as follows: 95°C for 30 seconds followed by 16 cycles of 95°C for 30

seconds, 55°C for 60 seconds, 68°C for 12 minutes. Then the reaction was placed on ice for 2 minutes to cool the reaction to ≤37°C. 1 μl of the Dpn I restriction enzyme (10 U/μl) was added directly to each amplification reaction. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. The reaction mixtures were spun in a microcentrifuge for 1 minute and immediately incubated at 37°C for 1 hour to digest the parental supercoiled dsDNA. Then the transformation protocol (see 2.2.1.8) was performed.

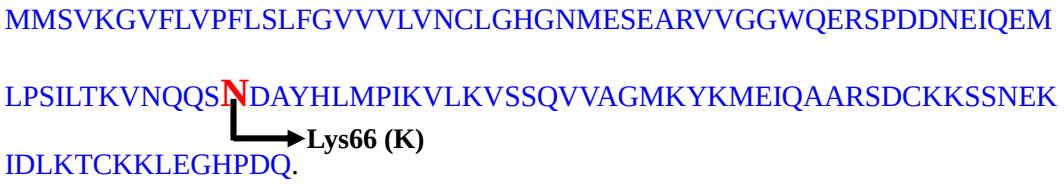


Fig 2.3 Schematic of CPI2 gene site direct mutation from Asn66 (N) to Lys66 (K).

2.2.1.8 Transformation of competent *E. coli*

The following procedure was used to transform host bacterial cells with all recombinant plasmids: The vial(s) containing the ligation reaction(s) or plasmid was centrifuged and placed on ice. 50 μl One Shot® Top10 or JM109 cells were used as hosts for the recombinant plasmids. For each ligation/transformation, 2 μl of each ligation reactions or plasmids were directly added and mixed gently into 50 μl competent cells and held on ice for 30 minutes. The suspension was then incubated for exactly 30 seconds at 42°C in a water bath before being returned to an ice bath. 250 μl of pre-warmed S.O.C medium were added to the bacterial suspension which was incubated in a shaker at 37°C for exactly 1 hour. After this incubation period, 100 μl of the transformed cell suspension was applied to LB agar plates which were then inverted and incubated at 37°C. Following overnight incubation individual colonies were selected for analysis by agarose gel electrophoresis (0.8-1% agarose, in 0.5× TBE buffer, 120 V for 40-50 minutes). Colonies containing the required plasmid were grown on for plasmid isolation, PCR, or sequencing (see below).

2.2.1.9 Small scale preparation of plasmid DNA

Small scale plasmid preparation was carried out according to the manufacturer's instructions of the QIAprep spin Miniprep kit. Transformed *E. coli* cells that harbor the plasmids of interest were grown in LB containing antibiotic (50 μg ml^{-1} ampicillin for pcDNA3.1- plasmids or 10 μg ml^{-1} kanamycin for pET- expression plasmids) at 37°C for 16 hours in a shaking incubator. Bacteria from 10 ml of cultures were collected by centrifugation for 5 minutes at 10,000 g. The pelleted bacterial cells were resuspended in 250 μl of Buffer P1 and then 250 μl of Buffer P2 were added and mixed by gentle inversion of the tube 4-6 times. Then, 350 μl of Buffer N3 were added and again mixed by gentle inversion 4-6 times. The culture was then centrifuged for 10 minutes at maximum speed in tabletop microcentrifuge and the supernatant fluid obtained (about 850 μl) applied to the QIAprep column. Unbound material was removed from the QIAprep column by centrifugal (1 minute at 10,000 g on a microcentrifuge) washing with 750μl Buffer PE. The QIAprep column was then placed in a clean 1.5 ml microcentrifuge tube, and the bound DNA was eluted by addition of 50 μl buffer EB (10mM Tris-CL, pH 8.5). The columns were allowed to stand for 2 minutes before eluted DNA was recovered by centrifugation for 1 minute. Eluted plasmid DNA was stored at -20°C.

2.2.1.10 Sequencing and analysis of double-stranded plasmid DNA

Plasmid DNA was prepared for sequencing according to the instructions of BigDye® XTerminator™ Purification Kit (Applied Biosystems). To 2 μl DNA templates were added: 3 μl distilled water; 2 μl 5× sequencing buffer (Bigdye terminator); 1μl sequencing primer (usually T7 and/ or BGH, for pcDNA3.1-, pDEC- and pISO- plasmids; T7 and/ or T7 terminator for pET- plasmids); Bigdye solution 2 μl (total volume 10μl). Reactions were performed through 25 cycles of 95°C for 30 seconds, 50°C for 20 seconds, and 60°C for 4 minutes, and stopped by cooling to 4°C or placing on ice.

Samples were sent to a specialist unit at the University of Edinburgh (The GenePool) for

sequencing. Sequence editing was carried out with the DNASTAR's Lasergene sequence analysis software, run on Windows XP. Sequence alignments were performed using MegAlign program in DNASTAR's Lasergene. Sequences were extracted from the NCBI database. Sequence comparisons with the national center of biotechnology information (NCBI) database were performed using BLAST NCBI online program.

2.2.1.11 Large scale purification of plasmid DNA

Cultures were initiated by addition of 50 µl *E. coli* carrying target plasmids into 500 ml of LB broth containing 50 µg ml^{-1} ampicillin, and the suspension incubated at 37°C for 16 hours. After this period the culture was centrifuged (6000 g, 30 minutes) at 4°C and the supernatant fluid discarded. The bacterial pellet was resuspended in 10 ml Buffer P1 to which was then added 10 ml Buffer P2. The suspension fluid was mixed thoroughly by vigorously inverting the sealed tube 4-6 times and then incubated at room temperature (15–25°C) for 5 minutes. Then, 10 ml of chilled Buffer P3 was added, mixed immediately and thoroughly by vigorously inverting the tube 4-6 times, and the suspension fluid was incubated on ice for 20 minutes. The suspension fluid was then centrifuged at ≥20,000 g for 30 minutes at 4°C and the supernatant fluid containing plasmid DNA recovered. This supernatant fluid was further clarified by a second centrifugation at ≥20,000 g for 15 minutes at 4°C.

For purification of the plasmid DNA from the supernatant fluid, a QIAGEN-tip 500 column was equilibrated by washing, under gravity, with 10 ml Buffer QBT. The supernatant fluid containing the plasmids was then run through the QIAGEN-tip column under gravity. The column was then washed with 2 ×30 ml Buffer QC. Next, the DNA was eluted with 15 ml Buffer QF. The eluted fluid was collected into a 50 ml tube in which the DNA was precipitated by addition of 10.5 ml (or 0.7 volumes) isopropanol at room-temperature. Precipitated plasmid DNA was recovered by centrifugation at ≥15,000 g for 30 minutes at 4°C. The DNA pellet was then washed with 5 ml 70% ethanol at room-temperature and again collected by centrifugation at ≥15,000 g for 10 minutes. The washed pellet was then

air-dried for 5-10 minutes before being redissolved in TE buffer, pH 8.0. The yield of DNA was determined by UV spectrophotometry at 260 nm and quantitative analysis on an agarose gel. All plasmids were stored at -20°C.

2.2.2 Methods for protein analysis

2.2.2.1 Protein expression

2.2.2.1.1 Recombinant protein expression in *E.coli*

The recombinant of pET21b-(ALT/CPI2/VAH/TPX), pET29c-(ALT/CPI2/VAH/TPX), pET24a –(ALT/CPI2/VAH/TPX) and pET30a-(ALT/CPI2/VAH/TPX) containing a T7 tag at the N terminus and His tag at the C terminus (Appendix 5) were produced in bacteria BL21(DE3) (Novagen, UK). The transformed bacteria were cultured at 37°C until the absorbance at 600 nm reached 0.6, then expression of recombinant protein was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The bacteria were then recovered by centrifugation at 6000 g for 30 minutes. The pellets were weighed and then resuspended in His-tag binding buffer at a concentration of 1 gram of cells per 5 ml buffer. They were then incubated for 20 minutes at room temperature and resuspended in 50 ml of ice-cold His-binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole) at a concentration of 1 gram of cells per 5 ml buffer, followed by sonication on ice with 30 cycles (30 seconds on, 30 seconds off) at 23 KHz (MSE sonicator). The sonicate was clarified by centrifugation at 6000 g for 30 minutes. The supernatant fluid was collected before running the purification program 3 on AKTA system in Rick Lab. All buffers (1× Charge Buffer, 1× Strip Buffer, 1× Binding Buffer and 1× Elution Buffer) were prepared and connected to the right pipeline separately. The presence of recombinant protein was determined by SDS-PAGE and Western blotting. Protein concentration was determined by the Bradford method using protein assay dye reagent (Bio-Rad) with calibration using bovine serum albumin (Sigma-Aldrich) serial dilution starting with 2mg/ml concentration (see 2.2.2.4). Purified fractions were then dialyzed against phosphate buffered saline (PBS) for 2 days with 2 replacements of PBS and aliquoted at -20°C.

2.2.2.1.2 Protein expression in COS7 cell

Recombinant proteins were also produced using the eukaryote COS7 expression system. The COS7 cells were obtained from Sigma, UK and maintained by passage as follows: complete RPMI1640, distilled PBS and trypsin were prepared and warmed at 37°C before operation. Cells were checked under a microscope, until confluence was about 95% and the cells were healthy. The cells were gently washed with 7 ml PBS followed by the addition of 2 ml trypsin enzyme, then incubated at 37°C for 5 minutes. Cells were transferred from the flask to a tube and 0.2 ml cell liquid per 30 ml medium was added. Cells were incubated at 37°C for 24-72 hours for use of plasmid expression *in vitro.*

Two reaction mixtures were prepared in Eppendorf tubes for each transfection. In tube 1, 1.25 μg plasmid DNA was mixed with 500 μl Opti-MEM I (Invitrogen). In tube 2, 2μl lipofectamine was added to 500 μl Opti-MEM I. After 5 minutes, the DNA and lipofectamine solutions were mixed in a single Eppendorf tube and allowed to react further. After a further 20 minutes, the entire 1000 μl transfection mixture was added to the 3 ml COS7 cell cultures (in RMPI 1640 containing 10% FCS), which were then incubated for 5 hours at 37 °C. After this period, the cells were washed twice by centrifugation at 1100 g for 1 min with PBS. They were then resuspended in 3 ml RPMI 1640 (without FCS or antibiotics) and incubated for a further 48 hours at 37°C. The culture supernatant fluid containing expressed recombinant protein was collected by centrifugation at 1100 g for 5 minutes. The protein concentration was determined by the Bradford method using protein assay dye reagent (Bio-Rad), and then stored at -80°C.

This procedure was also used to prepare recombinant proteins from: pcDNA3.1-ALT, pcDNA3.1-ADDALT, pDEC-ADDALT, pISO-ADDALT, pcDNA3.1-CPI2, pcDNA3.1-CPImu, pcDNA3.1-VAH, pcDNA3.1-TPX, pDEC-CPI2, pDEC-CPImu, pDEC-VAH, pDEC-TPX, pISO-CPI2, pISO-CPImu, pISO-VAH, and pISO-TPX.

2.2.2.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Commercial kits (Invitrogen, UK) were used for SDS-PAGE. The manufacture's instructions were followed throughout. Protein samples (including recombinant proteins ALT, CPI2, VAH and TPX) were mixed with an equal volume of 2× SDS-PAGE sample buffer (Invitrogen, UK) and boiled for 10 minutes before being applied to the gels. Electrophoresis was performed at 200 volts (constant voltage) for 1 hour using the MiniPROTEIN system (Bio-Rad). Gels were stained for 10 minutes with Coomassie blue solution and destained 5 times for about 1 hour using SDS Gel destain solution before transfer directly on to the nitrocellulose filters for Western blotting.

2.2.2.3 Western blotting

Proteins were transferred from the gel to cellulose nitrate membranes for Western blotting by electrophoresis at 30 volts for 1 hour. After transfer the membranes were washed twice for 10 minutes with 15 ml 1× TBS and then incubated for 1 hour in 5% skimmed milk in TBS to block all remaining protein binding sites. This was followed by two washes of 20 minutes with 1× TBSTT (4 ml Triton-x, 1 ml Tween-20 in 1 litre TBS) and a single wash for 10 minutes with 15 ml 1×TBS. The membrane was then incubated with either His-Tag monoclonal antibody (Novagen, UK) or polyclonal antisera against individual recombinant proteins (ALT, CPI2, VAH and TPX) from mice diluted in 5% skimmed milk in TBS blocking buffer. After 1 hour incubation, the membrane was washed twice for 20 minutes in 1× TBSTT, then for 10 minutes with 1×TBS. The membrane was then incubated with goat anti-mouse IgG alkaline phosphatase conjugate (Invitrogen, UK) diluted (1:5000) in blocking buffer for 8 hours. The membrane was washed 5 ×5 minutes with 1×TBSTT and then incubated with the substrate (specify) Chemi Glow (luminol/enhancer solution: stable peroxide buffer =1: 1) (Alpha, USA) until the color developed and then the reaction stopped by absorption the rest of substrates. The results were recorded using the Gel Image system (Bio-Rad).

2.2.2.4 Determination of protein concentration

The protein concentration was determined by Coomassie (Bradford) Protein Assay (Pierce) on dialyzed samples. The assay was performed in duplicate with the dialysis buffer as a control, 8 serial dilutions of (top concentration of 2 mg ml $^{-1}$) BSA as the standard, and the plate was read at 540 nm.

2.2.3 In vitro lymph node cell culture

Lymph nodes recovered from mice and washed in RPMI 1640 were crushed between two pieces of autoclaved filcotex/ Nitex plate and then diced with scissors in 500 μl of RPMI 1640 in a petri dish. The larger pieces of lymph node were allowed to settle and then the supernatant fluid collected and transferred to a 15 ml Falcon tube with RPMI 1640 added to a volume of 5 ml. Cells were spun down at 1100 g for 5 minutes, followed by resuspension in 5 ml RPMI 1640. Cells were counted and resuspended at 5×10^6/ml. 3 ml of RBC Lysis Buffer (Sigma) were added and mixed well after discarding the supernatant. After a further 4 minutes, 10 ml of RPMI 1640 were added and the cells were spun down at 1100 g for 5 minutes, followed by a finally resuspension in 10 ml RPMI 1640. Cells were counted and resuspended at 10^7/ml. Each well of 96 well plates was dispensed with cells 100 μl of cells (5×10^5/well). Cultures were incubated at 37°C in 5% CO_2 incubator.

2.2.4 Cell proliferation assays

For *in vitro* cell proliferation, lymph node cells were cultured in triplicate with *L. sigmodontis* antigen (final concentration 10 μg/ml), RPMI 1640 and anti-CD3 (final concentration 1 μg/ml) in a final volume of 200 μl. Cultures were incubated at 37℃ in 5% CO_2 for 48 hours followed by addition of 10% of total volume Alamar Blue (Invitrogen, UK) and incubated for another 24 hours. Plates were read at 540 nm for proliferation. Then the plates were spun down at 1100 g for 2 minutes, and the supernatant fluid was harvested carefully by avoiding the cells. The supernatant fluid was kept at -20℃ for further cytokines analyses.

2.2.5 Cytospin assay

Cytocentrifuge preparations from 200 µl cells (see 2.2.7) from the pleural cavity (2×10^6 cells/ml) in PBS were made using a Shandon Cytospin or Cytospin 4 (Thermo) at 200 g for 5 minutes. The slides were dried at room temperature overnight, then the slides were stained with Diff-Quick (Dade) according to the manufacturer's instructions. Briefly, Quick-Diff FIX was added to the slides 10 times, 1 second each followed by Quick-Diff RED 6 times,1 second each and Quick-Diff BLUE 10 times ,1 second each. Once the slides had dried, DPX mountant for histology (Sigma) was used to seal the slides with small cover slips. The cell numbers and types were determined by microscopic examination of at least 300 cells per slide.

2.2.6 Enzyme linked immuno-sorbent assay (ELISA)

2.2.6.1 IgG assay

Specific anti-*L. sigmodontis* IgG1 and IgG2a responses were measured by indirect ELISA against whole soluble extract coated at 10 mg/ml, and anti-LsALT1, anti-LsCPI2, anti-VAH or anti-TPX antibodies against the respective recombinant proteins coated at 50 µl per well at 5 mg/ml by indirect ELISA. Briefly, after antigen coating, plates were blocked with 200 µl PBS + BSA 4% per well. After washing with TBST solution, 50 µl of serially diluted serum were added (starting at 1: 400, serial dilution 1:2) when measuring titres, or at 1/800 when O.D. were used, and incubated overnight. Biotinylated detection antibody diluted in PBS+BSA 1% (IgG1: 1/6000 (goat anti mouse, Adsorbed Southern Biotech, Cat no 1070-05), IgG2a:1/4000 (goat anti mouse, Adsorbed Southern Biotech, Cat no 1080-05) was added for 2h at room temperature, and wells were incubated in 50%TMB-50% H_2O_2 (KPL) and stopped with addition of 25 µl of 1 mM H_2SO_4 when the blanks developed blue. Plates were read at 450 nm within 10 minutes of acid addition. Antibody titres were determined as the highest dilution factor for which O.D. values exceeded 3 standard deviations above control

wells on the same plate.

2.2.6.2 IgE determination

Total IgE production throughout all experiments was measured by sandwich ELISA. 96 wells plates were coated with IgE capture antibody (rat anti mouse, clone R35-72, BD Pharmingen, Cat No. 553413) diluted in carbonate buffer at 2µg/ml. After 4 times wash with TBST, plates were blocked with 100µl per well 5% skimmed milk carbonate buffer, and incubated with serum samples (1:100) or standards (purified mouse IgE, BD Pharmingen, Cat no. 557079, top standard concentration at 5µg/ml, diluted serially 1:2) at 37°C for 2 hours. 50µl per well detection biotinylated antibody (rat anti mouse IgE, BD Pharmingen, Cat no. 553419) was added after washing at 2µg/ ml in 0.5% FCS-TBST after which 100µl Extravidin Peroxidase (1/8000, cat: E2886, Sigma, UK) and incubated at 37°C for 30 minutes, then 100µl TMB substrate per well were added after 5 times plates wash. The reaction was stopped with 50µl 1 mM H_2SO_4 per well, and plates were read in a spectrophotometer at 450nm.

2.2.6.3 Cytokine detection

Cytokines of IL4, IL5, IL10, IFNγ and IL13 were detected by sandwich ELISA as follows. 96 wells plates were coated at 50 µl/well with capture antibody [all cytokines were tested with ELISA kits, IL4 kit (cat: KMC0041, Invitrogen UK); IL5 kit (cat: CMC0053, Invitrogen UK); IL10 kit (cat: CMC0103, Invitrogen UK); IL13 kit (cat: CMC2223, Invitrogen UK); IFNγ kit (cat: CMC4033, Invitrogen UK), respectively in carbonate buffer (the dilution factor was 1:500 for IL4, IL10, IFNγ and 1: 250 for IL5 and IL13)]. Plates were incubated at 4°C overnight. Plates were lavaged 3 times with TBST followed by incubation in PBS + 4%BSA (200µl / well) for 2 hours at room temperature in the dark. Then 50 µl / well 2-fold diluted standard (top concentration: IL4 at 8 ng/ml, IL5, IL10, IL13 at 10 ng/ml, IFNγ at 50 ng/ml diluted with PBS-BSA 1% for IL4, IL5, IL10, IFNγ, and dulbecco's modified eagle medium (DMEM) -5% FCS for IL13) was added in duplicate after wash,

followed by the addition of 50 µl / well sera samples, and then incubated at 4°C overnight. Plates were lavaged 3 times and incubated in biotinylated antibody (final dilution: IL4, IL5, IFNγ at 1 µg/ml , IL13 and IL10 at 2 µg/ml, dilution buffer for IL4, IL5, IFNγ and IL10 was PBS-BSA 1%, for IL13, TBST- 5% FCS) for 1 hour at room temperature followed by 30 minutes incubation in AMDEX streptavidin-peroxidase (cat: E2886, Sigma, UK) with dilution 1: 6000 in PBS-1%BSA (IL13 in TBST-5% FCS) after lavaging 3 times. 50 µl/well TMB (cat: 002023, Invitrogen UK) was added and incubated in dark at room temperature. When blue was intense only in upper standard wells, the reaction was blocked by addition of 20 µl per well H_2SO_4 at 1 mM. Plates were read at 450 nm.

2.2.7 Immunization and Necropsy

For immunization, female BALB/c mice, 6-8 weeks old, were divided into groups of 6 animals. Each mouse was vaccinated by inoculation of 40 µg plasmid plus adjuvant (pIL4 or pIL4+pFlt3L+pMIP1α) into the tibialis muscle. After vaccination, electroporation (ECM 830, BTX, Harvard Apparatus) was performed with parameters of 100 v/cm, 8 pulses, 20.4 miliseconds per pulse length and 460 miliseconds interval between pulses. Challenge infections of 25, 30 or 40 L3s were administered 4 weeks after the second dose. The time interval between boots was 4 weeks. Necropsy was performed 10 days or 60 days post challenge as follows: the mice were euthanized with Vetelar/ Domitor and the thoracic lavage cells were obtained by thorough washing of the pleural cavity with total 10 ml cool PBS. The supernatant fluid was separated and kept for cytokines detection and cytospin procedures. Worms were pooled into one tube and fixed with final 70% ethyl alcohol. The mediastinal and parathymic lymph node in thoracic cavity were removed for cell culture.

2.2.8 Larval, adult and Microfilariae counting

Worms were counted for measurement of protection. Intact worms were counted under microscope one by one. For the microfilariae counting, tube containing 30 µl mouse heart blood per 200 ml FACS lysing solution (cat no 349202, Becton Dickinson) was centrifuged,

then 120 µl supernatant fluid was dropped out. The rest pellet and solution in the tube was smeared onto slide and the Mf were counted. Protection rate was calculated based on the worms recovered from primary infection and vaccinated challenge group, respectively. The formula is: protection %= (average of primary infection-average of vaccinated challenge)/ average of primary infection ×100%.

2.2.9 Flow cytometry

Spleen cell suspensions were prepared for flow cytometry at 1×10^6 cells per well in supplemented RPMI 1640 medium, then were incubated at 4℃ for 5 minutes in CD16/32 blocking buffer (5µg/ml in 1:20 2% mouse serum with FACS buffer: PBS plus 0.5%BSA and 0.05% sodium azide, mainly to reduce the unspecific bounding by block the FcRs on the antibodies), followed by staining for 30 minutes at 4℃ in dark with antibodies at the appropriate dilution as determined by titration. The antibodies were generally directly fluorochrome conjugated or biotinylated. The antibodies included anti-F4/80-FITC (1:100); anti-SiglecF-PE (1:100); anti-CD4-PCP(1:100); anti-CD5-FITC (1:100); anti-B220-PCP (1;100); anti-CD25-PE (1:100) and anti-Foxp3-FITC (1:100), as well as the appropriate isotype control antibodies (IgG2a, K-FITC, IgG2a, K-PE, IgG2a, K- biotinylated). The cells were then washed 3 times in FACS buffers before acquisition and analysis.

2.3 Statistical analyses

For assessing the statistical difference between two groups, Mann Whitney U test which compares medians instead of means was used. When the P-values were less than 0.05, differences between groups were considered significant. The mean +/- SE is shown unless otherwise stated. All graphs were prepared using PRISM (version 4.0, GraphPad Software, Berkeley, CA). For the analysis of contributors of protection, differences between mouse groups were analyzed with generalized linear models (GLM) or regression linear model. When the P-values were less than 0.05, differences between groups were considered significant. The non parametric Kruskal-Wallis rank sum test followed by Dunn’s test for

multiple comparisons were applied for parasite counts for which a post-hoc test was required. A principal component analysis (PCA) was performed to reduce large numbers of immunological measurements into fewer independent components. Briefly, data was scaled to null mean and unit standard deviation, the broken stick model was then used to select interpretable principal components, the biological interpretations of which were based on the size of individual rotations.

Chapter 3-Enhancement of immunogenicity and vaccine potential of ALT and CPI2 via modification of gene structure

3.1 Introduction

Infections with filarial nematodes are still major causes of public health in tropical and subtropical areas. About 150-200 millions people in more than 80 countries are infected with onchocerciasis and lymphatic filariasis. This situation has already been shown to be associated with the capability of most helminths to modulate or suppress the host's immune system in such a way to tolerate the parasites for a long life span.

How do helminths inhibit host immune attack and prolong their survival? The answers still remain unclear because of the involvement of factors from hosts, parasites and environments. However, after decades of research, scientists have obtained some explanations. The observation of lymphocyte hypo-responsiveness which showed that lymphocytes isolated from infected people failed to respond to worm antigens (Ottesen EA *et al*., 1977), indicating the existence of immuno-suppression caused by parasites, this hypothesis was supported by similar findings in rodent models of filariasis (Weiss N using *Dipetalonema. viteae* 1978). Presently, the common view is that the suppression from filarial infection is associated with the presence of Mf in the bloodstream (Weller PF, 1978; Dalesandro DA, 1976; Lammie PJ, Katz SP, 1983a; Lammie PJ, Katz SP, 1983b; O`Connor RA *et al.*, 2000; O`Connor RA *et al.*, 2003) and other stages especially the infective larva stage (King CL, 2001; Semnani RT *et al.*, 2004; Babu S *et al.*, 2006) .

Helminths infections are found to have the ability to modulate immunity in both humans and animal models. Helminth induced T-cell hypo-responsiveness is partially caused by the modulation of dendritic cells (DCs) and macrophages. For example, activation of DCs can be

modulated by $CD4^+$ natural killer cells produced during schistosome infection (Speziali E *et al*, 2010). Production of Tr1 cells from human DCs was modulated by TLR2-dependent mechanism through a schistosomes-originated glycolipid. Moreover, T cell suppression could be modulated by schistosome infection (Olds GR, Ellner JJ, 1984).

Studies on mouse models using filarial parasites have shown that $CD4^+$ antigen specific T cell proliferations could be inhibited by helminth-induced alternatively activated macrophages which contributed to the protection of host from infection (Siracusa MC *et al*, 2008). In addition, granulocytes including eosinophils, mast cells, neutrophils and basophils, which were regarded as effectors, are now found to play a role as immuno-modulators (Cadman ET, Lawrence RA, 2010)

Helminth parasites can induce $CD4^+CD25^+$ regulator T cells and other types of regulatory T cells (for instance Tr1, $CD4^+Foxp3^+$ cells) which can control Th2 responses and pathology and influence helminth survival. IL10, which is produced by iTreg cells and non-T cells, plays an important role in regulation of pathology during infection. In addition, the Foxp3 Treg cells also play a key role in the down-regulation of immune responses (Mahanty S, Nutman TB, 1995; Bluestone JA, Abbas AK, 2003; Mittrucker HW, Kaufmann SH, 2004; Satogunia J *et al.*, 2005).

A significant number of worm-derived molecules with immuno-modulatory prosperities have been examed including carbohydrates [i.e.lacto-N-fucopentaose III, GalNAcβ (Fucα1-2 Fucα1-3) GlcNAc] (Thomas PG, Harn DA Jr, 2004), lipids and lipid-associated moieties (i.e. Schistosome-derived lyso-phosphatidylserine) which has the ability to induce Tr1 (Van der Kleij D *et al.*, 2002); nucleic acids which can activate DCs and C-type lectins (DC-SIGN) (Meyer S *et al*., 2005) which can interact with worms leading to the changes in immunological phenotype; the excretory/secretory proteins, for example, cystatins, which play a role in interfering with antigen presentation by monocytes (Hartmann S, Lucius R,

2003) and polyproteins, which cause polyclonal antibody from B cells with stimulation of IL4 (Tezuka H *et al.*, 2002).

The present study has focused on two proteins, ALT and CPI2, which have been reported to induce protective immune responses when used as vaccine candidates. More importantly, they were reported to inhibit the immune responses as mentioned in Chapter 1 (Gomez-Escobar *et al.*, 2005; Murray J *et al.*, 2005; Manoury *et al.*, 2001). The results presented are aimed at the capabilities of ALT and CPI2 to evoke protective responses after removal of their immunomodulatory properties.

3.2 Results

3.2.1 Isolation of ALT and CPI2 genes

ALT and CPI2 gene sequences from *L. sigmodontis* were downloaded from GeneBank (http://www.ncbi.nlm.nih.gov/). The RT-PCR and standard PCR techniques were used to isolate ALT and CPI2 genes from *L. sigmodontis* L3 larvae stage and adult worm using ALT-specific and CPI2-specific primers, respectively (see primer table in chapter 2). Electrophoresis shows that the right size of amplicon of ALT (444bp), ADDALT (306 bp) and CPI2 (543 bp) were obtained (Fig 3.1). The sequences obtained were analyzed using the MegAlign program in DNASTAR Lasergene 7.1 software to check their authenticities and the results are shown in Fig 3.2 and 3.3. Alignments with *B. malayi* ALT1 (U57547), ALT2 (U84723) and *W. bancrofti* (AF084553) show that Ls-ALT1 includes a variable acidic domain (aa 22-67). The programs of BLASTn using nucleotide sequence and BLASTp using amino acid sequences were used to identify the isolated Ls-ALT gene. BLASTn showed the isolate shares high similarities with Bm-ALT2 gene (XM-001896791) and *L. sigmodontis* abundant larval transcript-1 (ALT1) (DQ451171) (identities = 79% and 100%, respectively). BLASTp result showed the isolate shares high similarity (e value = 5e-80) with abundant larval transcript-1 protein (ABE02808), and belongs to the Chromadorea ALT superfamily,

suggesting the correct gene of ALT had been obtained.

In the case of CPI2, the BLASTn result showed the isolate shares high identities (99%) with *L. sigmodontis* Ls-cystatin precursor (AF229173), and the BLASTp results showed it shares high similarity (e value=5e-66) with Ls-cystatin precursor (AAF35896) and belongs to CY superfamily. The alignments with *B. malayi* (Bm-CPI-1, U80972), Bm-CPI-2 (AF015263), *O. volvulus* (Ov-CPI-1 AF177194; Ov-CPI-2, P22085), *L. sigmodontis* (Ls-CPI, AF229173), *A. viteae* (Av-CP, L43053), *N. brasiliensis* (Nb-CPI, AB050883), *H. contortus* (Hc-CPI, AF035945) and Chicken cystatin C (P01038) showed that conserved features SND, QVVAG and PW motifs are all present in Ls-CPI-1 and Ls-CPI-2, suggesting the correct gene of CPI had been obtained. Interestingly, the SND motif, reported to be present in Bm-CPI-2, not in Bm-CPI-1, was found in both the Ls-CPI-1 and Ls-CPI-2 genes.

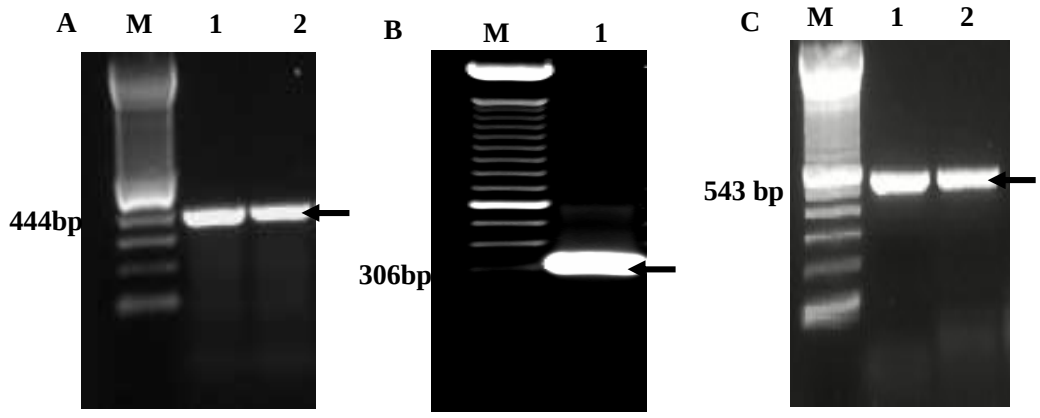
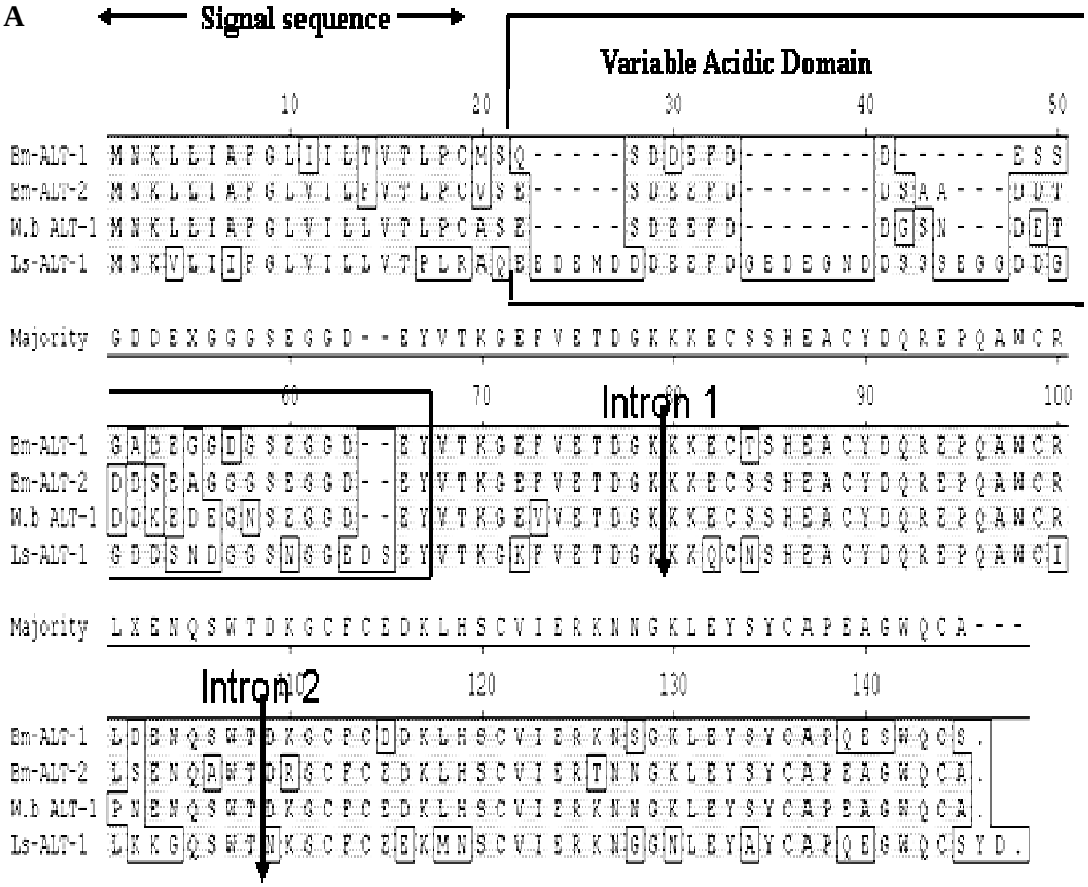


Fig. 3.1 Electrophoresis of RT- PCR amplification of ALT, ADDALT and CPI2 in 0.5% TBE solution at 120 V for 40 minutes. Adult worms from jird were used as source of mRNA. (A) PCR products of ALT. M. 100 bp ladder marker; 1, 2 ALT; (B). PCR products of ADDALT. M. 100 bp ladder marker; 1, ADDALT; (C) PCR products of CPI2. M. 100 bp ladder marker; 1, 2 CPI2;

A



B

Percent Identity

	1	2	3	4	5	6	7	
1		79.4	78.6	68.3	40.5	42.1	65.9	1
2	22.0		86.8	65.1	44.2	45.0	65.1	2
3	23.1	13.6		68.2	43.4	43.4	66.7	3
4	35.1	44.4	43.0		45.4	46.6	72.3	4
5	89.1	87.6	94.9	79.9		89.5	41.8	5
6	86.8	83.2	95.2	80.9	7.2		44.4	6
7	35.1	40.4	40.4	30.2	84.0	85.2		7
	1	2	3	4	5	6	7	

Divergence

1 Bm-ALT-1
2 Bm-ALT-2
3 W.b ALT-1
4 Ls-ALT-1
5 Ov-ALT-1
6 Ov-ALT-2
7 A.viteae ALT

C

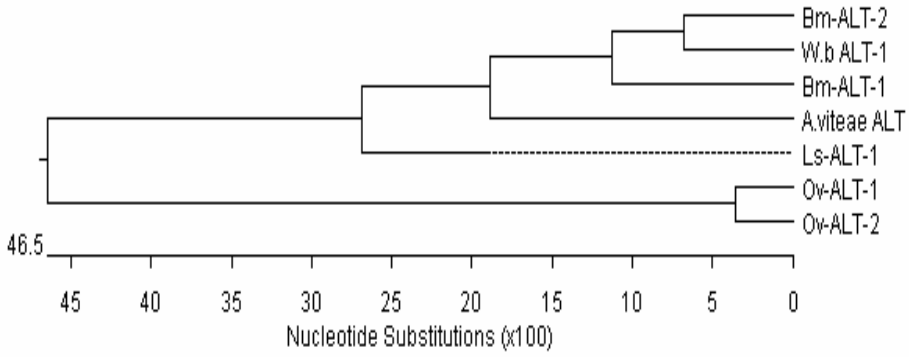
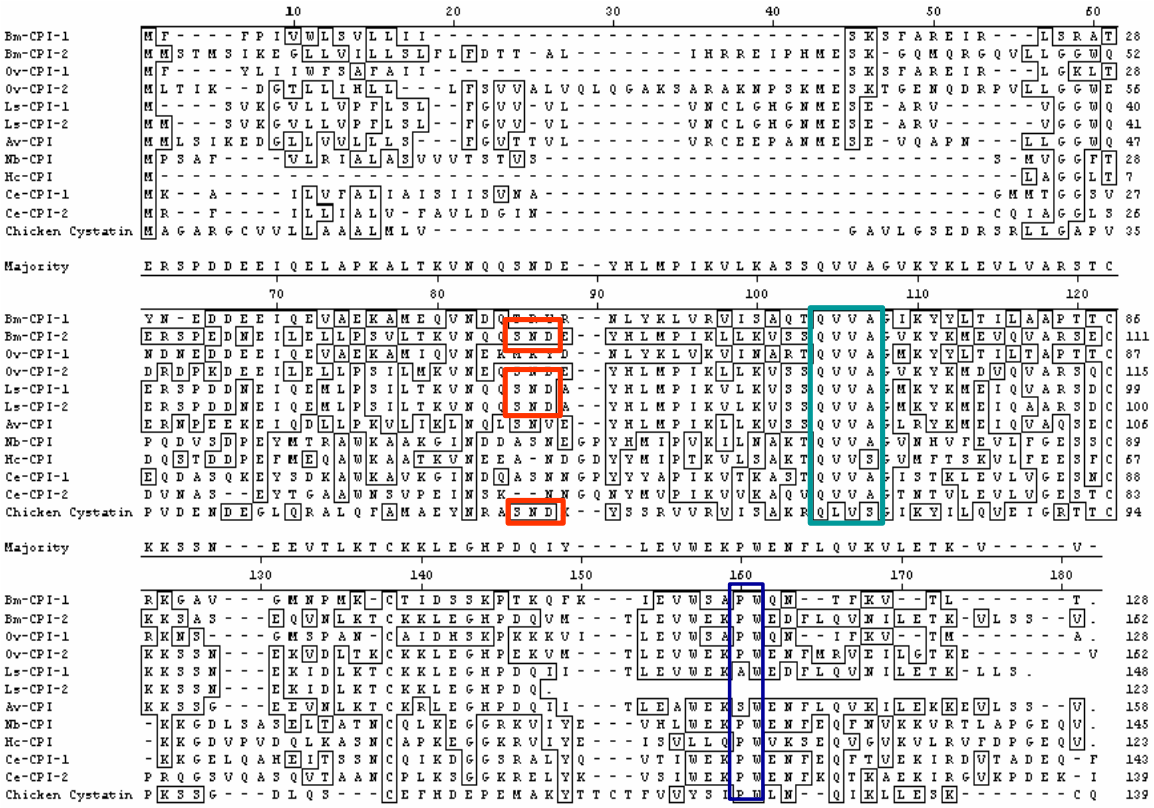


Fig. 3.2. Analysis of Ls-ALT protein using DNASTAR Lasergene 7.1 software. (A) Alignment of sequence of *L.sigmodontis* ALT-1 compared to *B. malayi* ALT , ALT2 and *W. bancrofti*. The intron position in *W. bancrofti* has not been determined. (B) Sequence distance result of alignment of ALT proteins. Clustal W in program MegAlign was used to determine alignment. Ls-ALT, *A. viteae* ALT, *B. malayi* ALT, ALT2 and *W. b*ancrofti, Ov-ALT-2 and Ov-ALT-1 were examined. (C). Outline of the phylogenetic trees of the ALT proteins based on the alignment outcomes.

A



B **Percent Identity**

Divergence

	1	2	3	4	5	6	7	8	9	
1		21.1	21.9	18.0	22.7	64.8	22.7	22.0	17.2	1
2	194.7		21.7	21.6	20.9	20.3	59.3	64.2	22.8	2
3	183.8	169.5		44.6	15.1	21.1	23.8	20.3	50.3	3
4	212.0	188.1	83.9		18.0	19.5	21.6	16.3	38.1	4
5	167.9	195.0	212.0	206.0		25.8	23.7	22.0	17.3	5
6	38.5	187.8	183.8	192.5	167.9		25.0	22.0	21.1	6
7	177.9	39.8	180.1	184.3	165.0	166.3		59.3	22.1	7
8	185.4	38.8	153.8	224.0	199.0	170.4	48.5		17.1	8
9	204.0	162.3	76.2	92.3	207.0	185.6	163.5	183.3		9
	1	2	3	4	5	6	7	8	9	

1 Bm-CPI-1
2 Bm-CPI-2
3 Ce-CPI-1
4 Ce-CPI-2
5 Chicken Cystatin
6 Ov-CPI-1
7 Ov-CPI-2
8 Ls-CPI-2
9 Nb-CPI

C

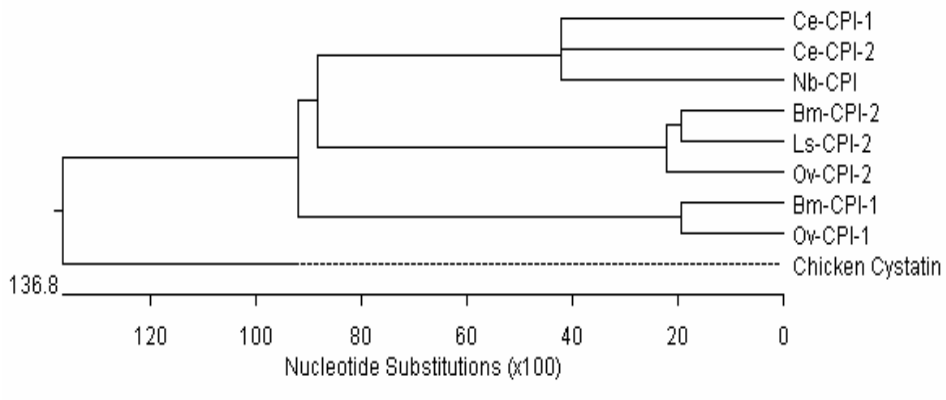


Fig.3.3 Comparison of *L. sigmodontis* and other cystatin sequences. (A) Sequence alignment of *L. sigmodontis* and other metazoan cystatins. The proposed AEP-binding loop residues (numbered 66–68) are outlined in red boxes. The cystatin-specific flexible loop, QVVAG in Ls-CPI-1 and Ls-CPI-2, is also shown. Accession numbers for cDNA or protein sequences are as follows: *B.malayi* – *Bm*-CPI-1; *Bm*-CPI-2; *O.volvulus* – *Ov*-CPI-1; *Ov*-CPI-2; *L. sigmodontis*–*Ls*-CPI; *A. viteae*– *Av*-CPI; *N. brasiliensis* – *Nb*-CPI; *H. contortus* – *Hc*-CPI; Chicken cystatin C. (B) Sequence distance result of alignment of ALT proteins. Clustal W in MegAlign program was used to construct alignment. *B. malayi* – *Bm*-CPI-1; *Bm*-CPI-2; *O. volvulus* – *Ov*-CPI-1; *Ov*-CPI-2; *L.sigmodontis*– *Ls*-CPI-2, *A. viteae* – *Av*-CPI; *N. brasiliensis* – *Nb*-CPI; Chicken cystatin C were applied to process. (C). Outline of the phylogenetic trees of the CPI proteins based on the alignment outcomes.

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3.2.2 Site-directed mutagenesis of Ls-CPI2 to Ls-CPImu

A site-directed mutagenesis of Ls-CPI2 gene which is the substitution of Asn66 with Lys66 was performed, this mutation was named Ls-CPImu (Fig. 3. 4). PCR identification and DNA sequencing showed it was mutated into Lys66 successfully. The DpnI enzyme was used to distinguish the methylate and unmethylated DNA.

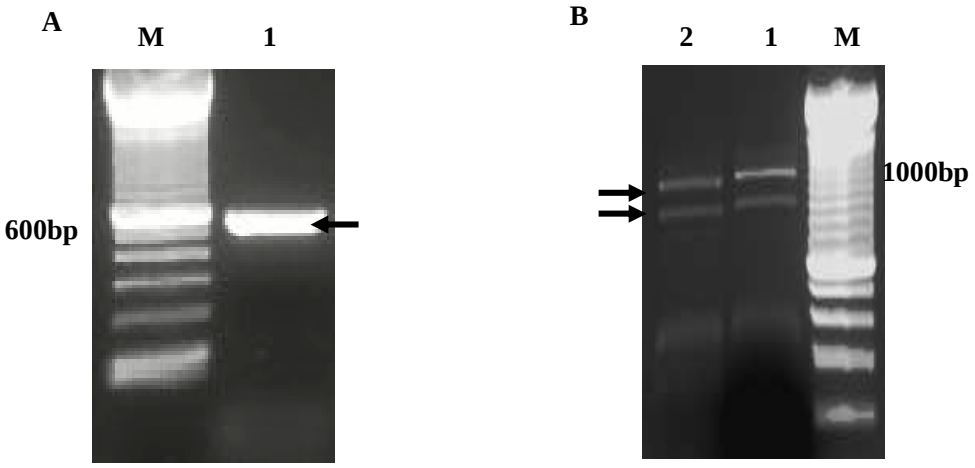


Fig.3.4 Agarose electrophoresis of site-directed mutagenesis of Ls-CPI2 in 0.5% TBE solution at 120 V for 40 minutes. (A) PCR amplification of pcDNA3.1-CPImu; (B) single digestion of pcDNA3.1-CPI2 with DpnI enzyme.

3.2.3 Construction of plasmids

PCR products of ALT, ADDALT and CPI2 for pcDNA3.1- construction were ligated into pcDNA^{TM}3.1Directional TOPO expression vector (5514 bp backbone length) according to the kit instruction. The pDEC205- plasmids contain the antibody scDEC205 which targets DEC205 receptor of DCs. Antibody pISO- plasmids were constructed as controls. Before the ligation into vectors, pDEC205-OVA (OVA gene size 1167bp) and pISO-OVA were double digested with NotI/ XbaI enzymes (Fig.3.5). Then, NotI/ XbaI-digested PCR products of ADDALT, CPI2 and CPImu were ligated into digested pDEC205- and pISO- to replace the OVA gene on the vector, respectively. Clones were confirmed by PCR using T7 and BGH primers, respectively (Fig. 3.6). Expression plasmids of pET29c-CPI2 and pET21b-ALT were constructed by the ligation of NdeI/ XhoI digested PCR products of CPI2 and ALT with

NdeI/ XhoI digested pET29c and pET21b, respectively. Clones were confirmed by PCR using T7 and T7 terminator primers. The plasmids of pcDNA3.1-ADDALT, pDEC205-ADDALT, pcDNA3.1-CPI2, pcDNA3.1-CPImu, pDEC205-CPI2, pDEC205-CPImu, pISO-ADDALT, pISO-CPI2, pISO-CPImu, pET29c-CPI2 and pET21b-ALT were sent for sequencing and results showed that all plasmids were constructed successfully.

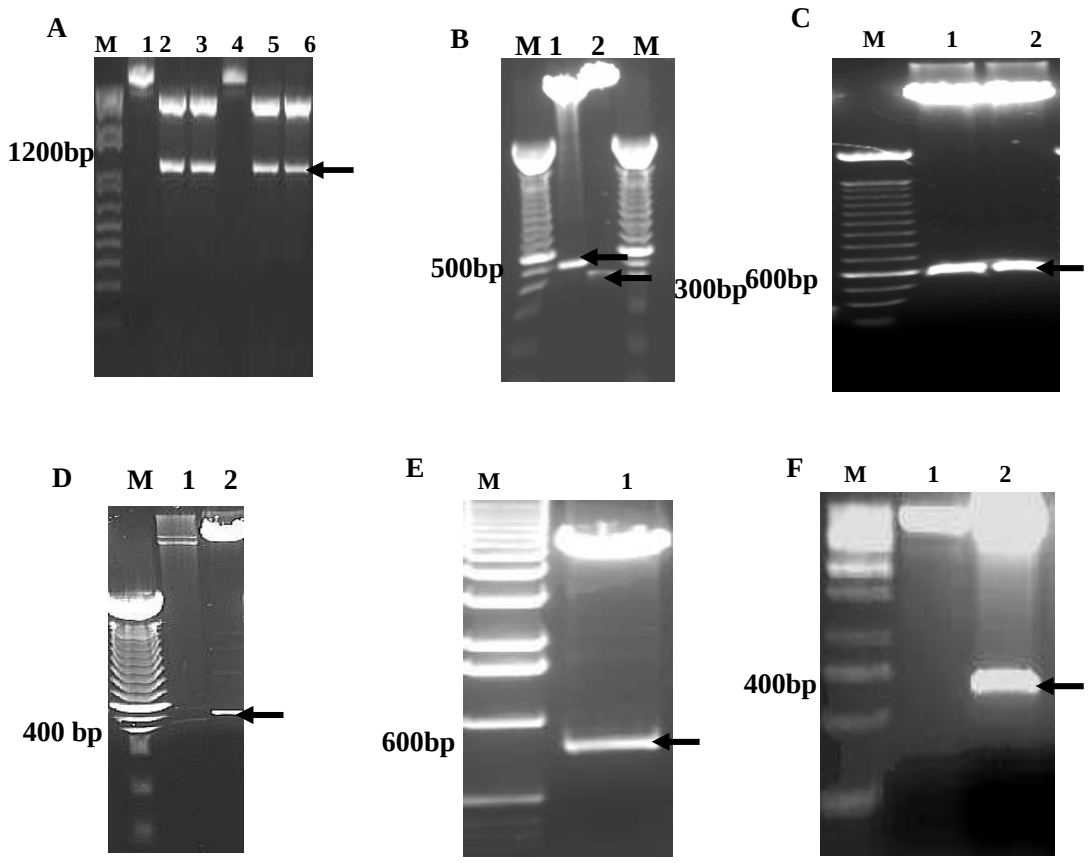


Fig. 3.5 Agarose electrophoresis of double digestion of plasmids for construction of vaccines in 0.5% TBE solution at 120 V for 40 minutes. (A) pDEC205-OVA and pISO-OVA using NotI/XbaI. M, 1kb marker. 1, undigested pDEC205-OVA. 2, 3, digested pDEC205-OVA. 4, undigested pISO-OVA. 5, 6, digested pISO-OVA; (B) pcDNA3.1-ALT and pcDNA3.1-ADDALT. M, 100bp marker. 1, digested pcDNA3.1-ALT. 2, digested pcDNA3.1-ADDALT with BamH I/ XbaI; (C) pcDNA3.1-CPI2 and pcDNA3.1-CPImu using BamH I/ XbaI. M, 100bp marker. 1, digested pcDNA3.1-CPI2. 2, digested pcDNA3.1-CPImu; (D) pDEC205-CPI2. M, 100bp marker. 1, empty pDEC205. 2, NotI/XbaI digested pDEC205-CPI2; (E) pET29c-CPI2 double digestion with NdeI/XhoI. M, 1000bp marker. 1, digested pET29c-CPI2. (F) pET21b-ALT double digestion with NdeI/XhoI. M, 100bp marker. 1, undigested pET21b-ALT 2, digested pET21b-ALT.

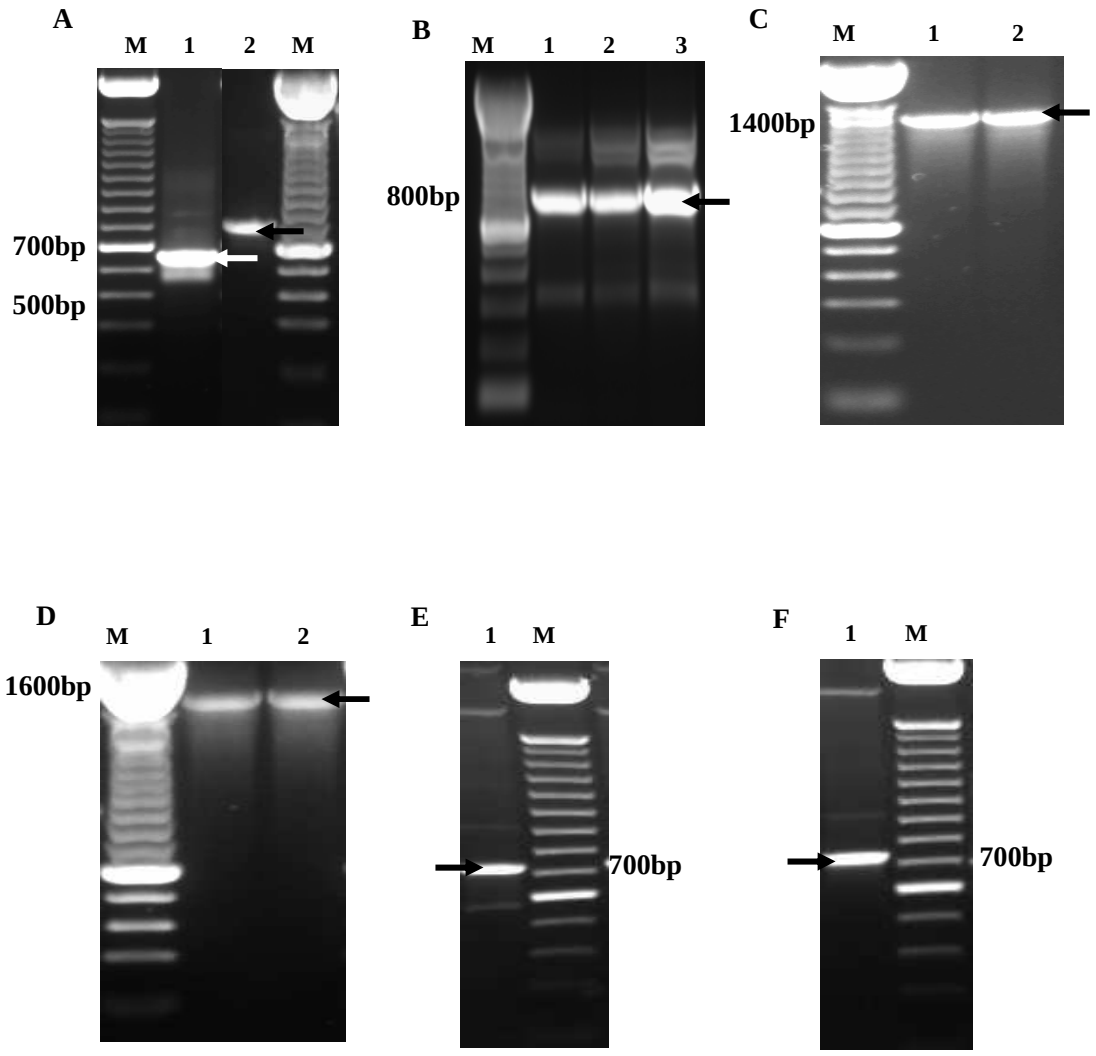


Fig.3.6 Electrophoresis of PCR identification of plasmids in 0.5% TBE solution at 120 V for 40 minutes.. (A).pcDNA3.1-ADDALT and pcDNA3.1-ALT using T7/BGH primers. M, 100bp marker; 1, pcDNA3.1-ADDALT; 2, pcDNA3.1-ALT (B) pcDNA3.1-CPI2 and pcDNA3.1-CPImu using T7/BGH primers. M, 100bp marker. 1, pcDNA3.1-CPI2. 2, 3, pcDNA3.1-CPImu; (C) pDEC205-ADDALT and pISO-ADDALT using T7/BGH primers. M, 100bp marker. 1, pDEC205-ADDALT, 2, pISO-ADDALT; (D) pDEC205-CPImu and pISO-CPI2 using T7/BGH primers. M, 100bp marker. 1, pDEC205-CPImu ,2, pISO-CPI2; (E) pET29c-CPI2 using T7/T7 terminator primers. M, 100bp marker. 1, pET29c-CPI2; (F) pET21b-ALT using T7/T7 terminator primers. M, 100bp marker. 1, pET21b-ALT.

3.2.4 Protein expression in *E. coli* and purification of recombinant antigen

To prepare antigens for ELISA, two genes, CPI2 and ALT, were sub-cloned into expression vector pET29c and pET21b, respectively, in which the recombinant protein carries a poly His tag. To determine the optimal expression, IPTG concentration (0.5 mM,1 mM, 2 mM), host strain (competent cell BL21-DE3 and Rosseta-gami 2), and temperature (22°C, 25°C, 30°C, 37°C) were tested. Pellets and supernatant fluids on 1, 2, 3, 4, 5 hour after IPTG induction were assessed by SDS-PAGE electrophoresis as shown in (Fig 3.7), which showed that the protein of CPI2 was expressed best at 37°C, 5 hour after 0.5 mM IPTG induction while ALT was expressed best at 37°C, 4 hour after 1 mM IPTG induction and the pellet contained most of the expressed proteins. The concentration of purified protein measured by Coomassie (Bradford) Protein Assay was 1 mg/ml for ALT and 0.217 mg/ml for CPI2.

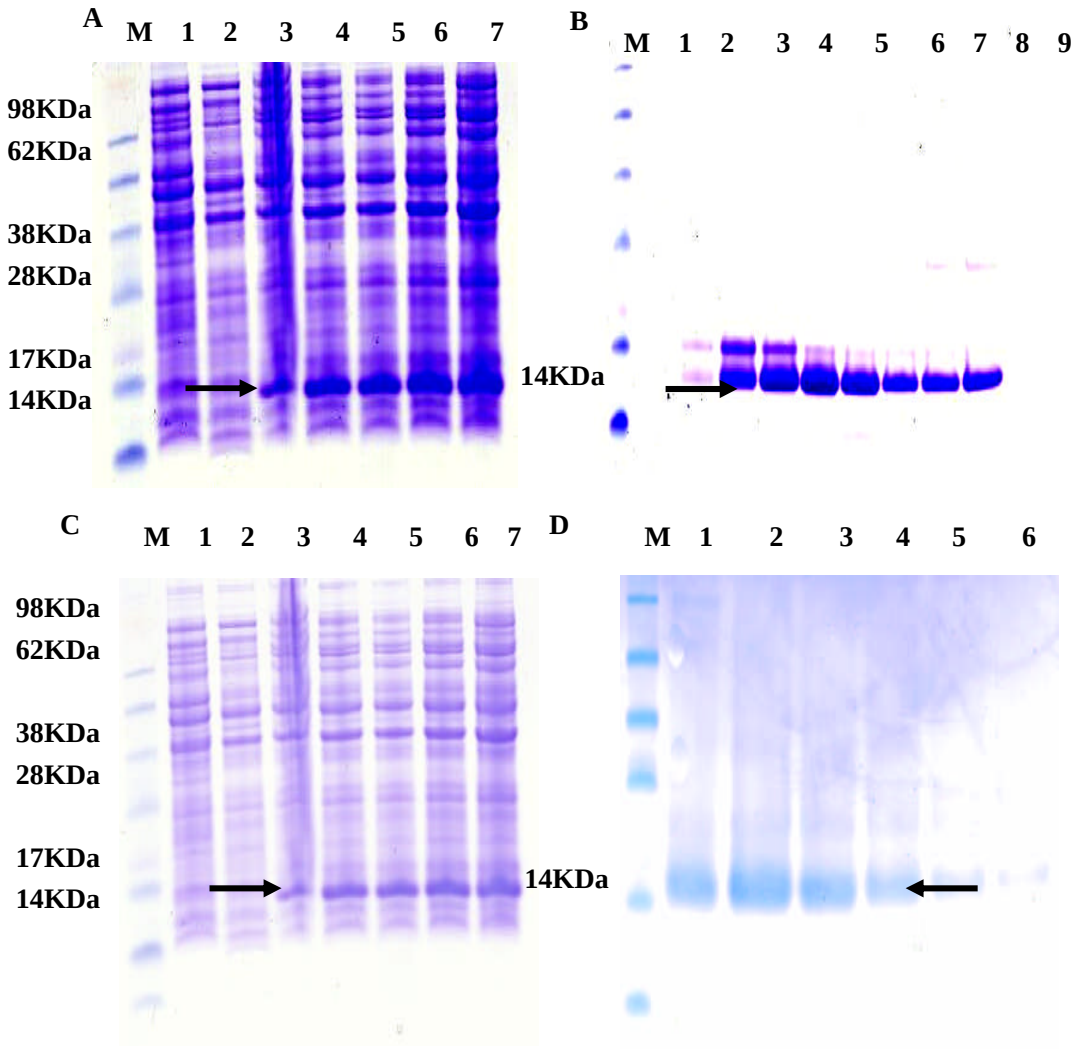


Fig. 3.7 SDS-PAGE electrophoresis of recombinant CPI2 and ALT at 200 V for 1 hour. (A). Protein expression of pET29c-CPI2 at 37℃ on different time points. M, See Blue plus 2 prestained standard(Invitrogen, UK). 1, uninduced empty pET29c. 2, uninduced pET29c-CPI2. 3, pET29c-CPI2 2 hour after 0.5mM IPTG induction. 4, pET29c-CPI2 3 hour after 0.5mM IPTG induction. 5, pET29c-CPI2 4 hour after 0.5mM IPTG induction. 6, pET29c-CPI2 5 hour after 0.5mM IPTG induction. 7, pET29c-CPI2 overnight after 0.5mM IPTG induction. (B). CPI2 protein eluted from His-binding column in the fractions. M, See Blue plus 2 prestained standard(Invitrogen, UK). 1-9: puritied fractions. (C). Protein expression of pET21b-ALT at 37℃ on different time points. M, See Blue plus 2 prestained standard(Invitrogen, UK). 1, uninduced empty pET21b. 2, uninduced pET21b-ALT. 3, pET21b- ALT 2 hour after 0.5mM IPTG induction. 4, pET21b-ALT 3 hour after 0.5mM IPTG induction. 5, pET21b-ALT 4 hour after 0.5mM IPTG induction. 6, pET21b-ALT 5 hour after 0.5mM IPTG induction. 7, pET21b-ALT overnight after 0.5mM IPTG induction. (D). ALT protein eluted from His-bounding column in the fractions. M, See Blue plus 2 prestained standard (Invitrogen, UK). 1-6: puritied fractions. Molecular Weight CPI2:11KDa; ALT: 14KDa

3.2.5 Identification of ALT and CPI2 by Western blotting

The ALT and CPI2 proteins expressed in *E. coli* were identified using Western blotting. Results showed that ALT / CPI2 protein from the pET21b / pET29C vector expressed in *E. coli* were recognized by the sera from mice immunized with plasmid pcDNA3.1-ALT and pcDNA3.1-CPI2, respectively (Fig 3.8).

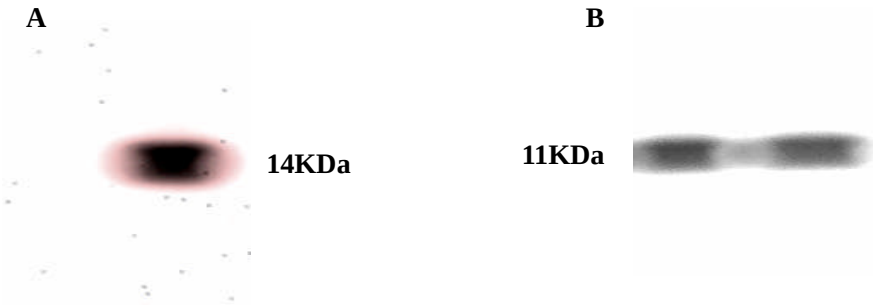


Fig. 3.8 Electrophoresis of Western blotting of ALT (A) and CPI2 (B) protein against sera from immunized mice with pcDNA3.1-ALT and pcDNA3.1-CPI2 vaccine, respectively at 30 V for 1 hour. Protein was separated under reducing conditions on a 10-12% acrylamide gel. Gel was probed with sera from immunized mice with pcDNA3.1-ALT or pcDNA3.1-CPI2 vaccine

3.2.6 Protein expression in COS7 cell and identification

1.25 μg DNA of each plasmid (pcDNA3.1-ALT, pcDNA3.1-ADDALT, pDEC-ADDALT, pISO-ADDALT which expressed an irrelevant antibody that did not bind to DCs, pcDNA3.1-CPI2, pcDNA3.1-CPImu, pDEC-CPI2, pDEC-CPImu, pISO-CPI2 and pISO-CPImu) was transfected into COS7 cells. Transfection remained for 48 hours, and supernatants for secreted protein and /or lysed cells were collected for protein harvesting. Reverse transcription PCR and Western blotting were carried out to identify whether the plasmid expressed in COS7 cell, or not. Results showed the transcripts of all plasmids were detected using ALT- or CPI2-specific primers (Fig. 3.9) and all proteins expressed in COS7 cell could be recognized strongly by the serum from the ALT- or CPI2 DNA immunized mice (Fig. 3.10).

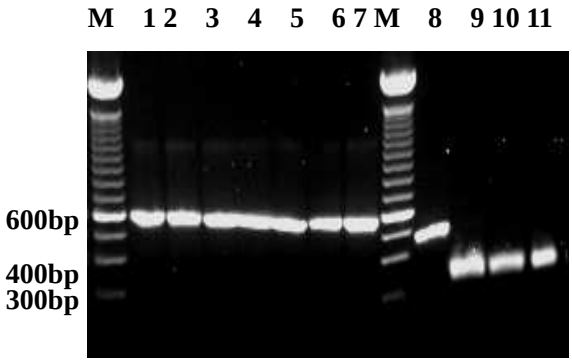


Fig. 3.9 Electrophoresis of RT-PCR amplification of gene expression in COS7 cells in 0.5% TBE solution at 120 V for 40 minutes. Plasmids of ALT, CPI2 and CPImu were transfected into COS7 cells in vitro, then cells were harvested and ultrasonicated, mRNA was extracted to for RT-PCR. M: 100bp DNA ladder; 1, 2: pcDNACPI2; 3: pcDNACPImu; 4: pDECCPI2; 5: pDECCPImu; 6: pISOCPI2; 7: pISOCPImu; 8: pcDNAALT; 9: pcDNAADDALT; 10: pDECADDALT; 11: pISOADDALT.

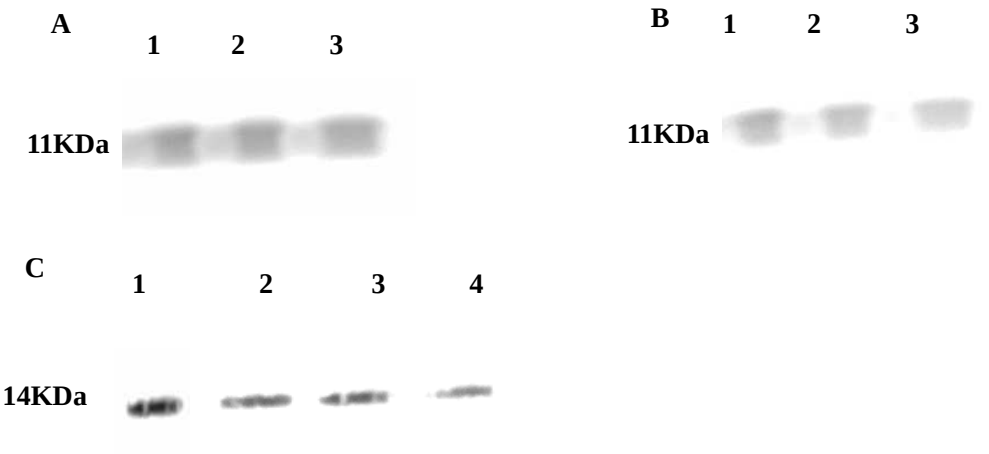


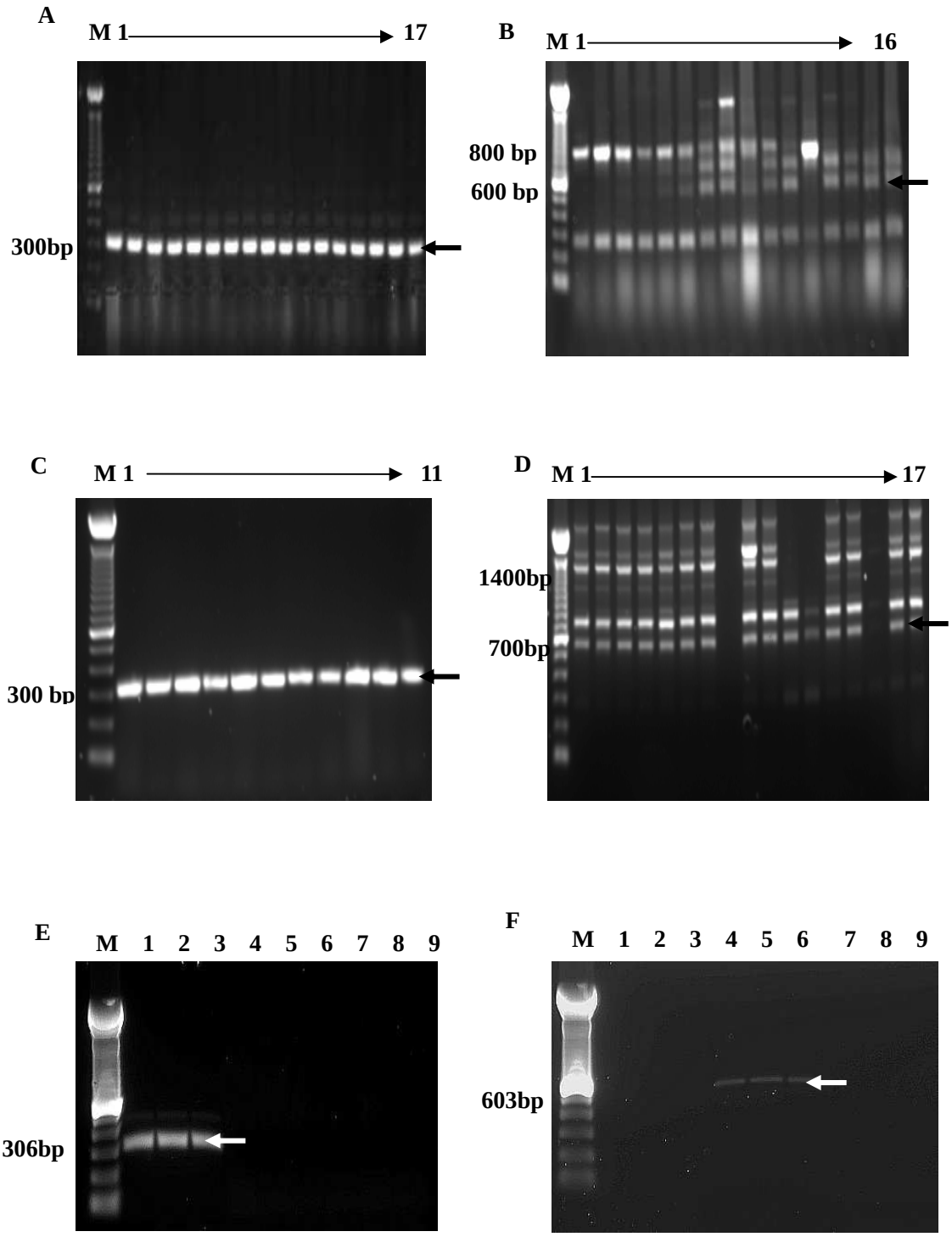
Fig. 3.10 Elctrophoresis of Western blotting of proteins expression in COS7 cells under reducing conditions on a 10-12% acrylamide gel at 30 V for 1 hour. Plasmids of ALT, CPI2 and CPImu were transfected into COS7 cells in vitro, then cells were harvested and ultrasonicated, and the supernatant fluids were used to perform the Western blotting against the serum from the ALT and CPI2 DNA vaccinated mice. (A). Western blotting of CPI2 and CPImu; 1, pcDNACPImu; 2, pDECCPImu; 3, pISOCPImu; (B). Western blotting of CPI2 and CPImu; 1, pcDNACPI2; 2, pDECCPI2; 3, pISOCPI2; (C). Western blotting of ALT and ADDALT; 1, pcDNAALT; 2, pcDNAADDALT; 3, pDECADDALT; 4, pISOADDALT.

3.2.7 Tissue distribution of ALT / CPI2 plasmids in mice

In order to test the efficiency and distribution of plasmids of ALT, ADDALT, OVA, CPI2 and CPImu in mice following the intramuscular injection and electroporation, two doses of

plasmid were given at 2 weeks intervals and necropsy was performed at 28 days after final immunization. Murine tissue was collected at the point of injection together with spleen, liver and lung and prepared for mRNA extractions. Reverse transcription PCRs with ADDALT- and CPI2-specific primers and T7 /BGH primers (which locate the flank of inserted gene on the plasmids, implying the existence of plasmids in tissues) were carried out. The results showed all genes were expressed in muscle, spleen, liver and lung when specific primers were used, but the OVA gene was not expressed in liver. All the positive PCR detections in muscle, spleen, liver and lung when T7/BGH primers were used showed the evidence of presence of plasmids (Table 3.1). The ALT/ADDALT vaccines were intensively distributed in muscle, spleen and lung, whereas most CPI2/ CPImu vaccines were expressed strongly in muscle, spleen and liver (Table 3.1).

Real time PCR was applied to test the quantity of genes expressed in local muscle. All vaccines were expressed in different levels, but the single chain DEC205- carried ADDALT and CPI2 vaccines expressed at higher levels than the others.(Fig 3.12). Fig 3.11 shows four examples for the tissue distribution *in vivo*.


A
M 1
17
300bp
B
M 1
16
800 bp
600 bp
C
M 1
11
300 bp
D
M 1
17
1400bp
700bp
E
M 1 2 3 4 5 6 7 8 9
306bp
F
M 1 2 3 4 5 6 7 8 9
603bp

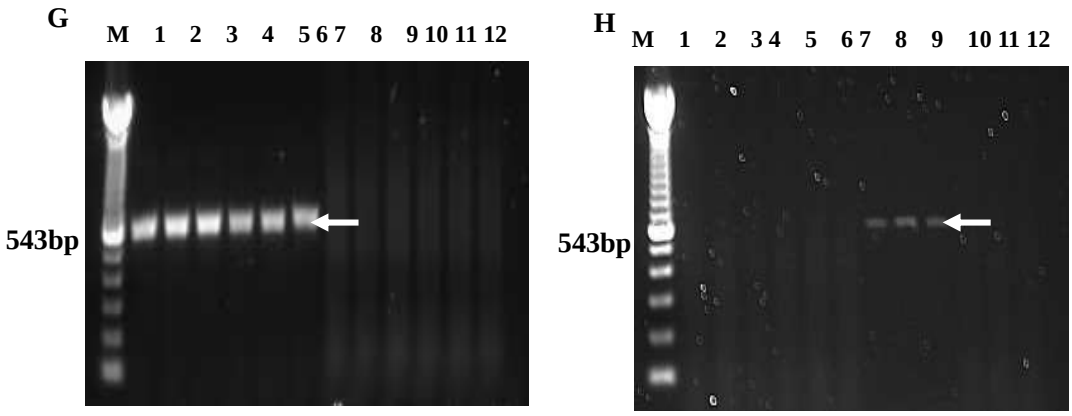


Fig.3.11 Electrophoresis of tissue distribution of ALT/CPI2 plasmids in mice in 0.5% TBE solution at 120 V for 40 minutes. All plasmids of ALT, ADDALT, OVA, CPI2 and CPImu were injected into muscle twice followed by the electroporation, 28 days later, muscle, spleen, liver and lung were taken to detect the gene distribution by specific primers and T7/ BGH primers by reverse transcriptional PCR. (A). ALT/ADDALT distribution in spleen detected by ADDALT-specific primers. 1-5: pcDNA-ADDALT; 2-10: pDEC-ADDALT; 11-15: pISO-ADDALT; 16-17: pcDNA-ALT; (B). ALT/ADDALT distribution in lung/liver detected by T7/BGH primers. 1-5; pcDNA-ALT in lung; 2-10: pcDNA-ADDALT in lung; 11-16: pcDNA-ADDALT in liver; (C). CP2/CPImu distribution in muscle detected by CPI2-specific primers.1-5: pcDNA-CPI2; 6-10: pDEC-CPImu; 11: pcDNA-CPImu; (D). CP2/CPImu distribution in muscle detected by T7/BGH primers.1-5: pcDNA-CPI2; 6-10: pcDNA-CPImu; 11-15; pDEC-CPImu; 16-17: pcDNA-CPI2; (E). ADDALT distribution detected by ADDALT-specific primers. 1-3: ADDALT; 4-6: OVA; 7-9: Naïve; (F). OVA distribution detected by OVA-specific primers.1-3: ADDALT; 4-6: OVA; 7-9: Naïve (G). CP2/CPImu distribution detected by CPI2-specific primers.1-3: CPI2; 4-6: CPImu; 7-9: OVA; 10-12: Naïve; (H). OVA distribution in muscle detected by OVA-specific primers. 1-3: CPI2; 4-6: CPImu; 7-9: OVA; 10-12: Naïve.

Table. 3.1 Summary of tissue distribution of ALT, ADDALT, OVA, CPI2 and CPImu vaccines in mice detected by RT-PCR (for detection of expressed sequence) and standard PCR (for detection of existence of plasmids)

	Specific primers				T7/BGH primers			
plasmid	**Muscle**	**spleen**	**lung**	**liver**	**Muscle**	**spleen**	**lung**	**liver**
pcDNA-ADDALT	+++	++	++	+	+	+	++	++
pDEC-ADDALT	+++	++	++	+	+	+	++	++
pISO-ADDALT	+++	++	++	+	+	+	++	++
pDEC-OVA	+	++	+	-	+	+	+	+
pISO-OVA	+	++	+	-	+	+	+	+
pcDNA-ALT	+++	++	++	+	+	+	++	++
pcDNA-CPI2	+++	++	+	+++	+	+	+	+
pDEC-CPI2	+++	++	+	+++	+	+	+	+
pcDNA-CPImu	+++	++	+	+++	+	+	+	+
pDEC-CPImu	+++	++	+	+++	+	+	+	+
pISO-CPImu	+++	++	+	+++	+	+	+	+
pISO-CPI2	+++	++	+	+++	+	+	+	+
Naïve	-	-	-	-	-	-	-	-

“+”represents Expression positive;“-“ represents no expression

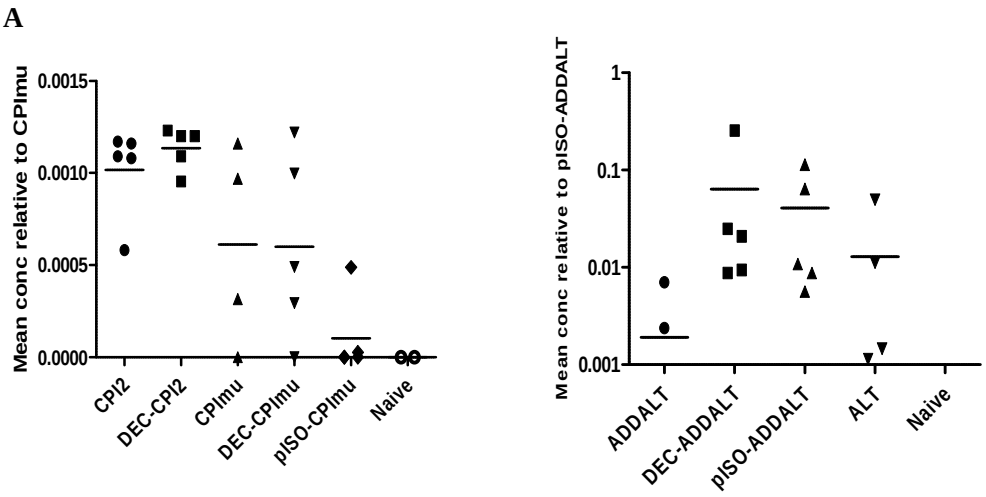


Fig 3.12 Relative quantification of ALT, ADDALT, CPI2 and CPImu vaccine in local muscle. All DNA vaccines were injected by the intramuscular route. 28 days later, local muscle was collected and real time PCR was applied to measure the quantification of each gene. (A). The relative quantity of CPI2/CPImu vaccines. (B). The relative quantity of ALT/ADDALT vaccines.

3.2.8 Stronger antibody responses were induced by DEC-ADDALT compared to control

In an attempt to enhance the immunogenicity of the ADDALT antigen, recombinant plasmid with the gene encoding an antibody with specificity for the DEC205 surface protein of dendritic cells was constructed. A fusion peptide consisting of a heavy and light chain of this antibody with the ADDALT peptide was encoded by the plasmid. Single chain antibody against DEC205 targets the antigen to the DCs via its surface receptor DEC205. pcDNA3.1-ADDALT, which is based on the backbone of vector pcDNA3.1 without the single chain antibody of DEC205, together with backbone plasmid alone (pempty) and pISO-ADDALT were injected as controls. In addition, the plasmids encoding IL4, MIP1α and Flt3L gene, respectively were used to enhance the host immune responses. Mice were given 3 inoculations at 14 days intervals. Challenge with 40 L3s was performed 28 days after the final vaccination and necropsy was carried out 60 days after challenge.

As shown in Fig.3.13, both pcDNA3.1-ADDALT and pcDNA3.1-DEC-ADDALT (designated as DEC-ADDALT) could induce much higher IgG1 antibody than the pempty control, but the DEC-ADDALT induced more IgG1 than pcDNA3.1-ADDALT. The same trend was seen with respect to IgG2a and IgE. In terms of cellular response, elevated levels of cytokines were detected together with eosinophils, neutrophils, macrophages, lymphocytes, monocytes and mast cells recruited into the pleural cavity compared to plasmid controls. However, there were no differences between pcDNA3.1-ADDALT and DEC-ADDALT (data not shown) for cellular responses.

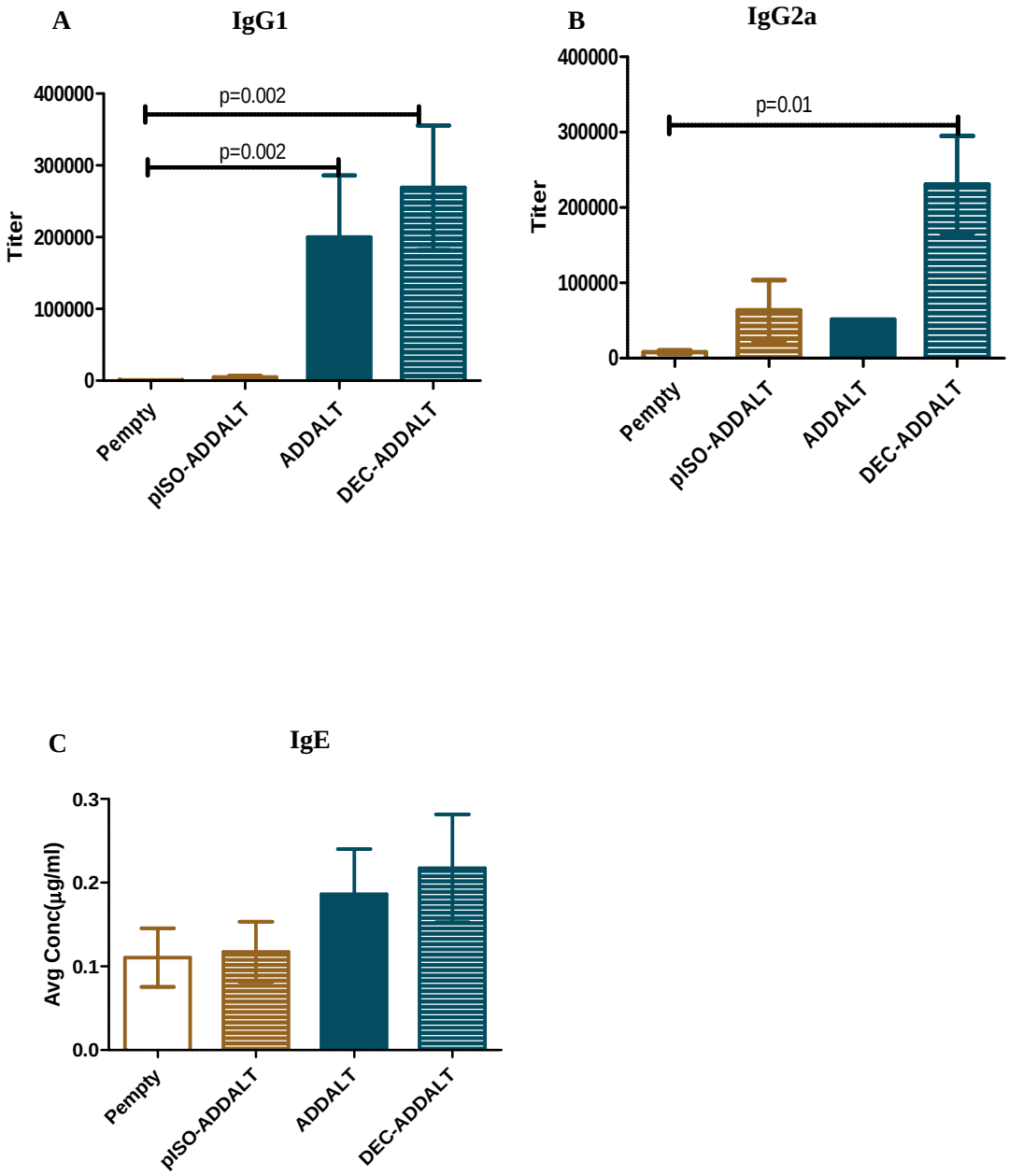


Fig. 3.13 Stronger antibody responses were induced by DEC-ADDALT compared to control. Antibody levels of IgG and IgE were compared between pcDNA3.1-ADDALT and DEC-ADDALT by indirect ELISA. Results are shown as titers of IgG and the mean of replicate samples (+/- S.E.M) for total IgE. 5 mice per group were tested. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

3.2.9 DEC-ADDALT provoked stronger Th2 antibodies

In order to test if DEC-ADDALT can provoke and augment the immune responses, three groups pISO-OVA, DEC-OVA and pISO-ADDALT were set up as controls for DEC-ADDALT, among them, pISO-OVA and pISO-ADDALT were set as controls to DEC-OVA and DEC-ADDALT, respectively. Mice were given 2 doses of DNA plasmid each plus a plasmid encoding IL4 as adjuvant followed by electroporation, on D0 and D21, mice were challenged with 40 L3s on Day 53. Blood was collected on D7, D14, D35 and D63 for the antibody dynamics. Necropsy was carried out 10 days post challenge (D63). For IgG1, there was a slight decline between D7 and D14 before an increase on D35 then was increased in mice vaccinated with DEC-ADDALT. The pattern of IgG2a followed a similar trend, although slightly higher levels of antibody against DEC-ADDALT were detected against pISO-OVA group (Fig 3.14, A, B). When IgG1 and IgG2a were measured on D10 post challenge (when the L3s migrate into the pleural cavity and moult to L4s), both showed an increased level. However, the titer of IgG1 was greater than that of IgG2a induced by DEC-ADDALT (Fig 3.14, C, D). Meanwhile, the level of IgE showed a significant enhancement in the DEC-ADDALT group with comparison with pISO-OVA control (Fig 3.14, E).

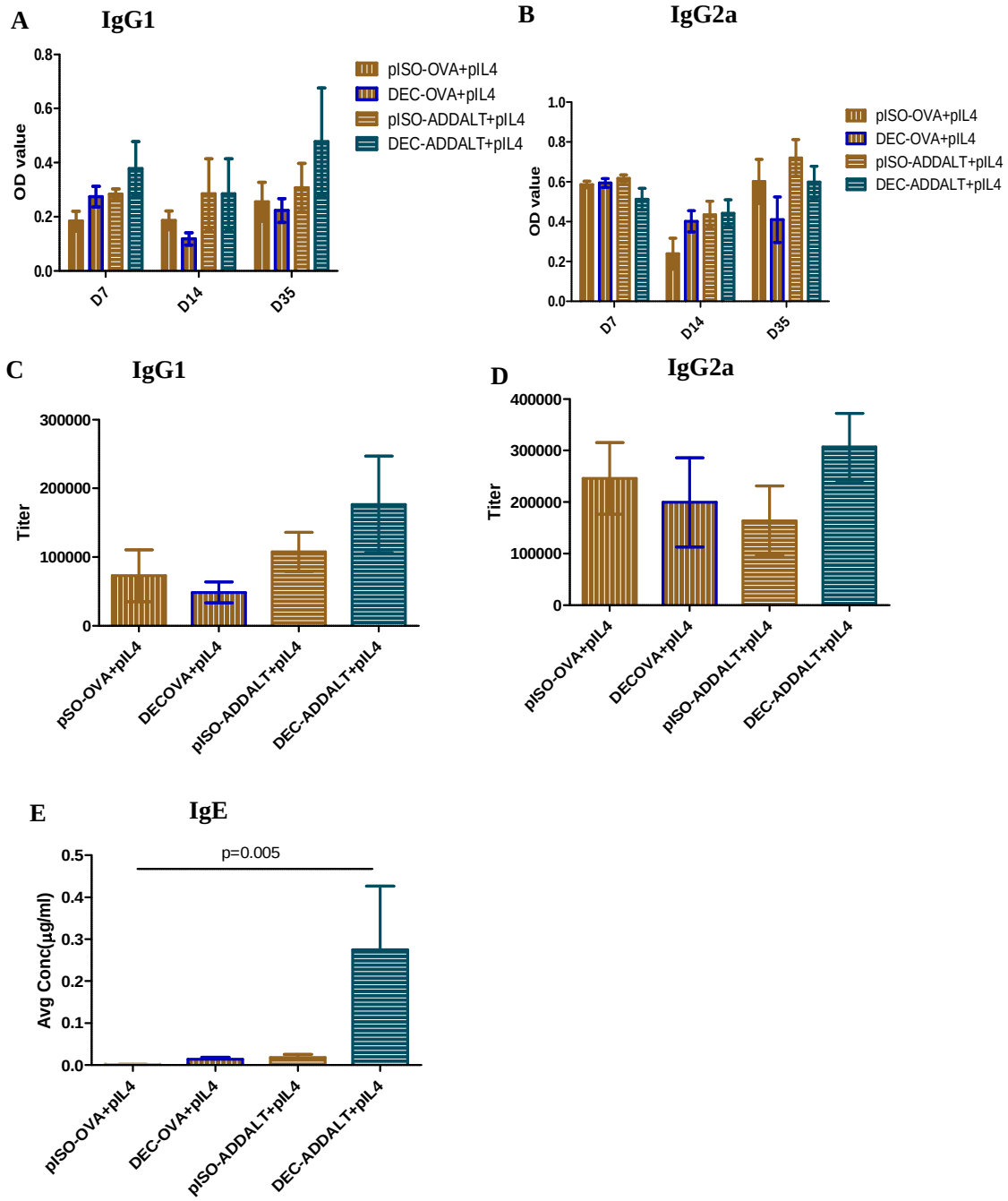


Fig 3.14 Stronger IgG1 and IgE level were elicited by ADDALT in BALB/c mice. 5 mice each group were given 2 doses of DNA plasmid each (pISO-OVA, DEC-OVA, pISO-ADDALT and DEC-ADDALT) with the adjuvant of IL4, at day 7, day 14 and day 35 post first injection, sera were tested for the IgG1 and IgG2a level by ELISA. Plates were coated with recombinant ALT protein. Mice were challenged 32 days after final immunization with *L. sigmodontis* L3, necropsy was performed 10 days post infection. IgG and total IgE were measured. (A). (B) IgG1 and IgG2a levels elicited by ADDALT at day7, 14 and 35. (C).(D).(E). IgG1, IgG2a and IgE level elicited by ADDALT 10 days post challenge. Results are shown as titer of IgG and the mean of replicate samples (+/- S.E.M) for total IgE. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyzed differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

3.2.10 DEC-ADDALT elicited Th2-biased cytokines

To investigate the cytokine profile produced by DEC-ADDALT vaccine, a capture ELISA was used to detect the expression of cytokines in the pleural cavity 10 days post challenge. Th2 cytokines IL4, IL5, IL13, IL10 and Th1 cytokine IFNγ were measured. Low levels of IL4 were detected in all samples with slightly greater amounts detected in the mice immunized with DEC-ADDALT. Low levels of IL5 were also detected with no difference between groups. However, IL13 levels were greatly increased in the group immunized with DEC-ADDALT. IL10 was undetectable. The expression of IFNγ showed an opposite profile to that of IL4 and IL13, only the controls not DEC-ADDALT induced high level of IFNγ (Fig.3.15).

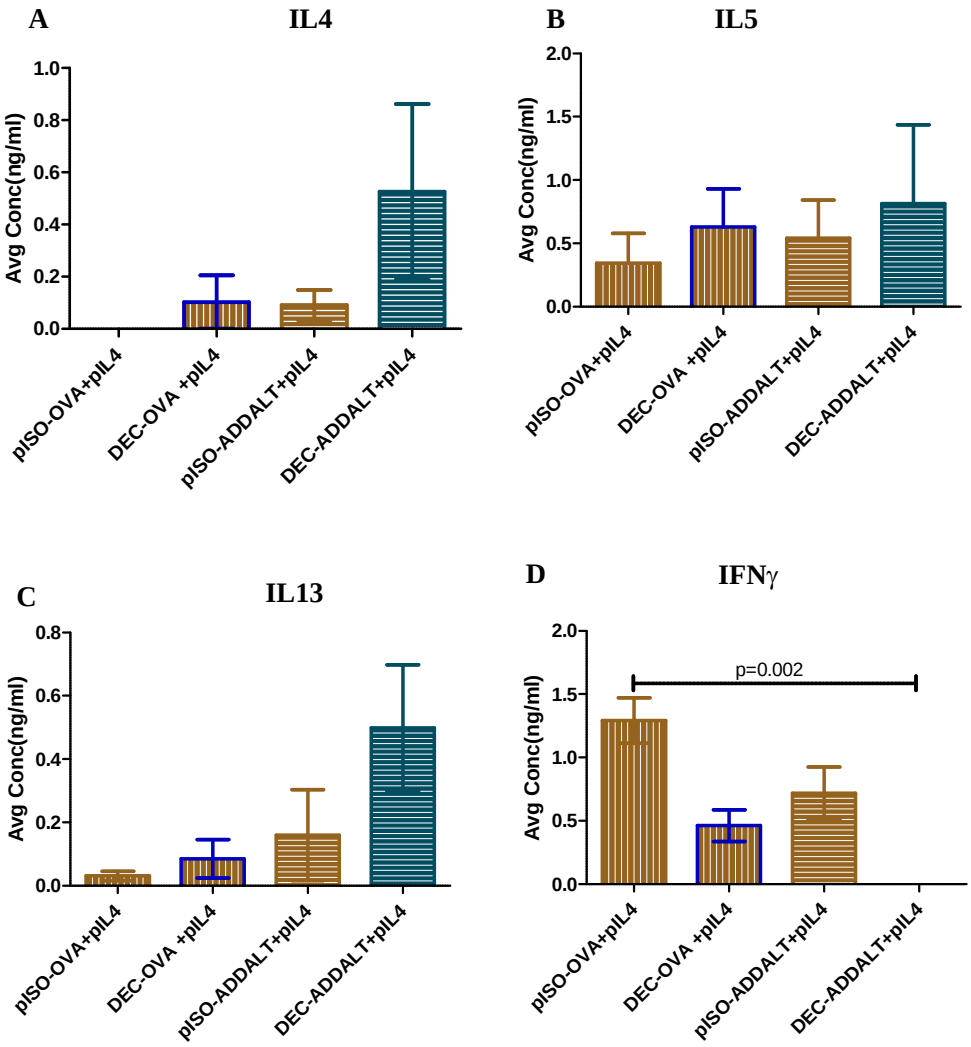


Fig.3.15 Th2-biased cytokines were induced by DEC-ADDALT in pleural cavity lavage. Supernatant fluids of pleural cavity lavage were harvested and the level of various cytokines (IL4, IL5, IL13 and IFNγ) were measured by capture ELISA - see methods and materials. Results are shown as the mean of replicate samples. 5 mice per group were tested. Standard curves were prepared with recombinant cytokine. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

3.2.11 Recruitment of total cells and eosinophils were decreased following vaccination with DEC-ADDALT

Cytospin analysis was used to investigate the type of cells recruited into pleural cavity 10 days after L3 infection. Results revealed lower total cell recruitment to the pleural cavity

following vaccination with DEC-ADDALT when compared with numbers recruited to the pleural cavity with vaccination of pISO-ADDALT. Similarly, the number of eosinophils recruited following DEC-ADDALT vaccination was decreased (not significantly) compared to that in the pISO-ADDALT group (Fig.3.16). The major cells recruited were the macrophages which represented about 60% of total cells and there was no difference among groups (Fig.3.16).

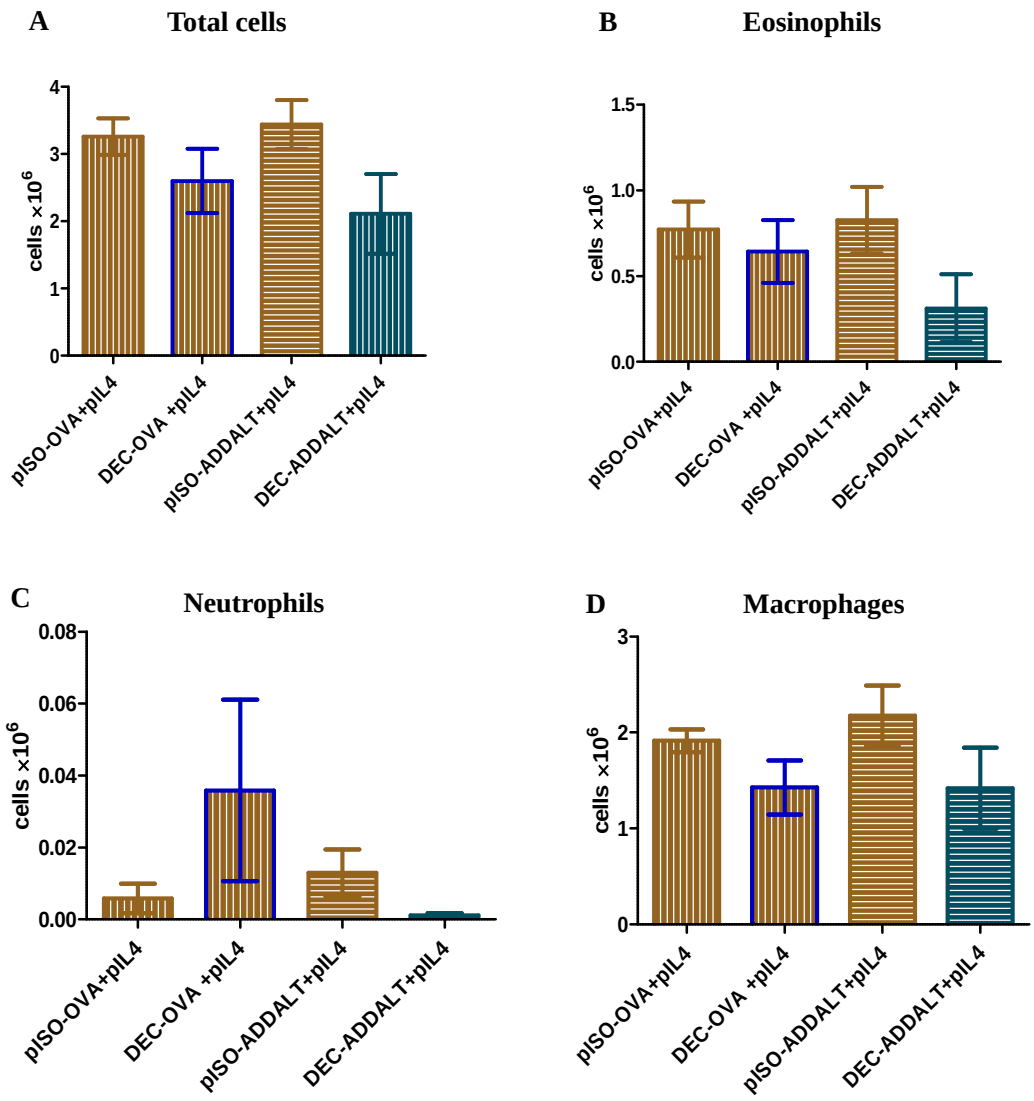


Fig. 3.16 Numbers of total cells and eosinophils decreased in pleural cavity following vaccination with DEC-ADDALT. Pellets of pleural cavity lavage were used for total cell counting on CASY model TT cell counter. Cells were used for the cytospin process and eosinophils and other types of cells were counted under a microscope on fixed slides, 300 cells minimum on each slide were counted. 5 mice per group were tested. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

3.2.12 Worm recovery was reduced but protection was not obtained following vaccination with DEC-ADDALT

The numbers of filariae in the pleural cavity lavage fluids were counted to evaluate the protective response provoked by the DEC-ADDALT vaccination. Mice were challenged with 40 L3s and at day 10 post challenge an average of 8 L3s was recovered in control groups immunized with pISO-OVA or pISO- ADDALT. In contrast, an average of 6 worms was recovered from the group immunized with DEC-OVA and an average of 4 worms was recovered from the group vaccinated with DEC-ADDALT. Although the numbers recovered from DEC-ADDALT vaccinated mice reduced compared to the controls of DEC-OVA and pISO-ADDALT, the difference among them was non-significant (Fig.3.17).

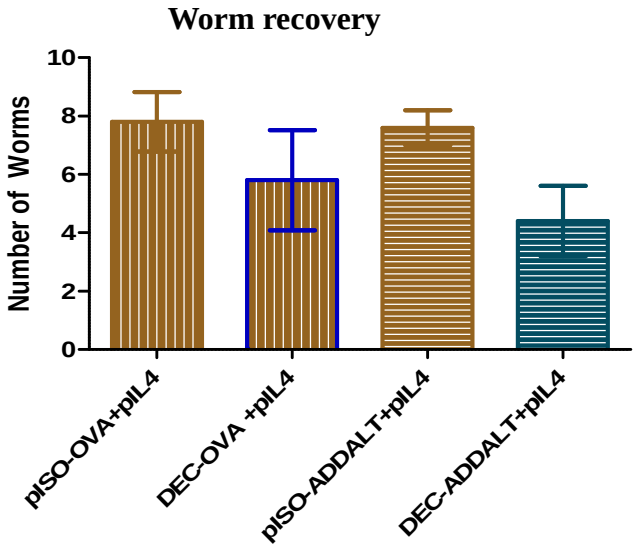


Fig.3.17 Worm recovery was reduced but protection was not obtained following vaccination with DEC-ADDALT was obtained. Necropsy was performed 10 day post challenge with 40 L3s, pleural cavity lavage fluids were harvested then filariae were counted under a microscope. 5 mice per group were tested. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

3.2.13 Immune responses induced by CPI2 was reversed by CPImu

The second candidate molecule for the DNA vaccine is CPImu, which is a mutation of the

CPI2 gene via the replacement of Asn66 (N) with Lys66 (K). To investigate the ability of CPImu to elicit a host immune response, three negative control groups (naïve, primary infection, pISO-OVA) were compared with BALB/c mice immunized with pDEC-CPI2 and pDEC-CPImu separately. All groups were also simultaneously inoculated with plasmids encoding IL4, MIP1α and Flt3L as adjuvants. Mice were given two inoculations with the recombinant plasmids followed by electroporation after each injection. Mice were challenged with 40 L3s 4 weeks post the final immunization which was performed 4 weeks post the first immunization and necropsy was performed 60 days after the challenge. Sera (blood), pleural cavity lavage and lymph nodes were collected. As shown in Fig. 3.18, pcDNA3.1-CPImu induced much stronger (not significantly) levels of IgG1 and IgG2a than pcDNA3.1-CPI2. Moreover, the IgG1level was enhanced greatly when pDEC-CPImu was compared with the pcDNA3.1-CPImu group, although the level of IgG2a in pDEC-CPImu was only slightly higher (not significantly) than pcDNA3.1-CPImu group. There was no enhancement of either IgG1 or IgG2a on pDEC-CPI2 vaccination in contrast to pcDNA3.1-CPI2 group, however, IgG1 level was significantly increased when pDEC-CPImu group was compared with pcDNA3.1-CPI2 and pDEC-CPI2, respectively. As for IgG2a, significant rise was only seen between pcDNA3.1-CPI2 and pDEC-CPImu. The significant stronger IgE response was only seen between pDEC-CPImu and pcDNA3.1-CPI2 group. Although there was no big difference between pcDNA3.1-CPI2 and pcDNA3.1-CPImu, much stronger IgE was produced by pcDNA3.1-CPImu immunization.

All data showed that the CPI2 gene either pcDNA3.1-CPI2 or pDEC-CPI2 could not induce strong immune responses, but such responses were reversed by the vaccination of CPImu, especially the pDEC-CPImu vaccination.

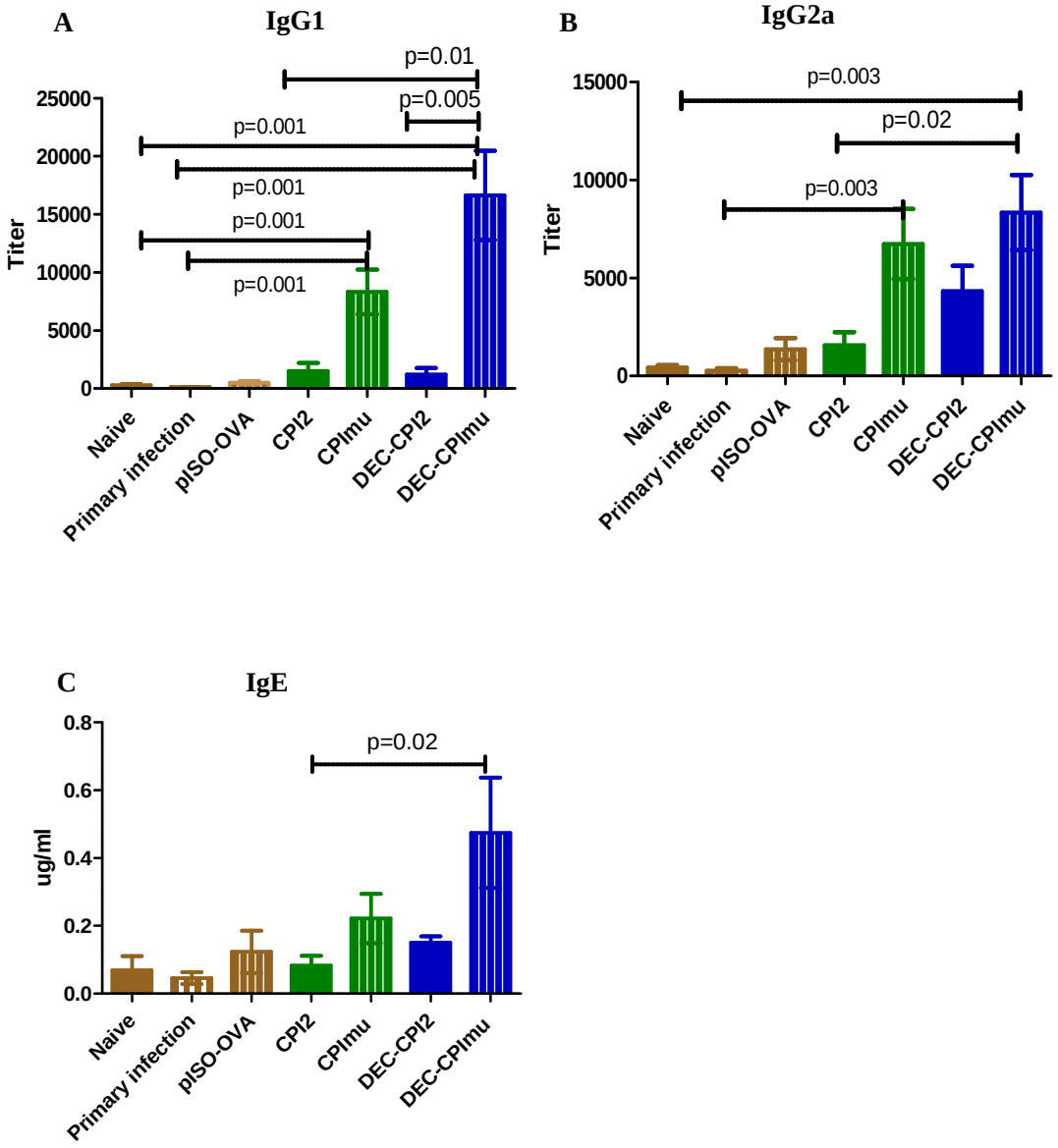


Fig. 3.18 Immune responses induced by CPI2 was reversed by CPImu. Antibody levels of IgG and IgE were compared among pcDNA3.1-CPI2, pcDNA3.1-CPImu, pDEC-CPI2 and pDEC-CPImu by indirect ELISA using recombinant CPI2 protein as the target (see methods and materials). All test groups were also inoculated with plasmids encoding IL4, Flt3L and MIP1α as adjuvants**.** Results are shown as titer of IgG and the mean of replicate samples (+/- S.E.M) for total IgE. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

3.2.14 DEC-CPImu vaccination induced a Th2 bias

IL4, IL5, IL10, IL13 and IFNγ were measured in pleural lavage fluid by the capture ELISA to evaluate the cellular immune effect of pDEC-CPImu vaccination. Results showed that high levels of IL4 could not be induced by the immunization of pcDNA3.1-CPI2 and pDEC-CPI2, but it could be elicited by pDEC-CPImu vaccination with a significantly stronger enhancement. Although the pcDNA3.1-CPImu and pDEC-CPI2 induced higher levels of IL5 than pcDNA3.1-CPI2, significant enhancement was seen on pDEC-CPImu vaccination with comparison to pcDNA3.1-CPI2. IL13 showed a similar profile to IL4 and significant greater production of IL13 was evoked by pDEC-CPImu immunization. However the profile of IL10 and IFNγ showed opposite trends. Only CPI2 (either pcDNA3.1-CPI2 or pDEC-CPI2) could induce high levels of IL10 and IFNγ, whereas the CPImu (both pcDNA3.1-CPImu and pDEC-CPImu) down-regulated the expression of IL10 and IFNγ (Fig.3.19).

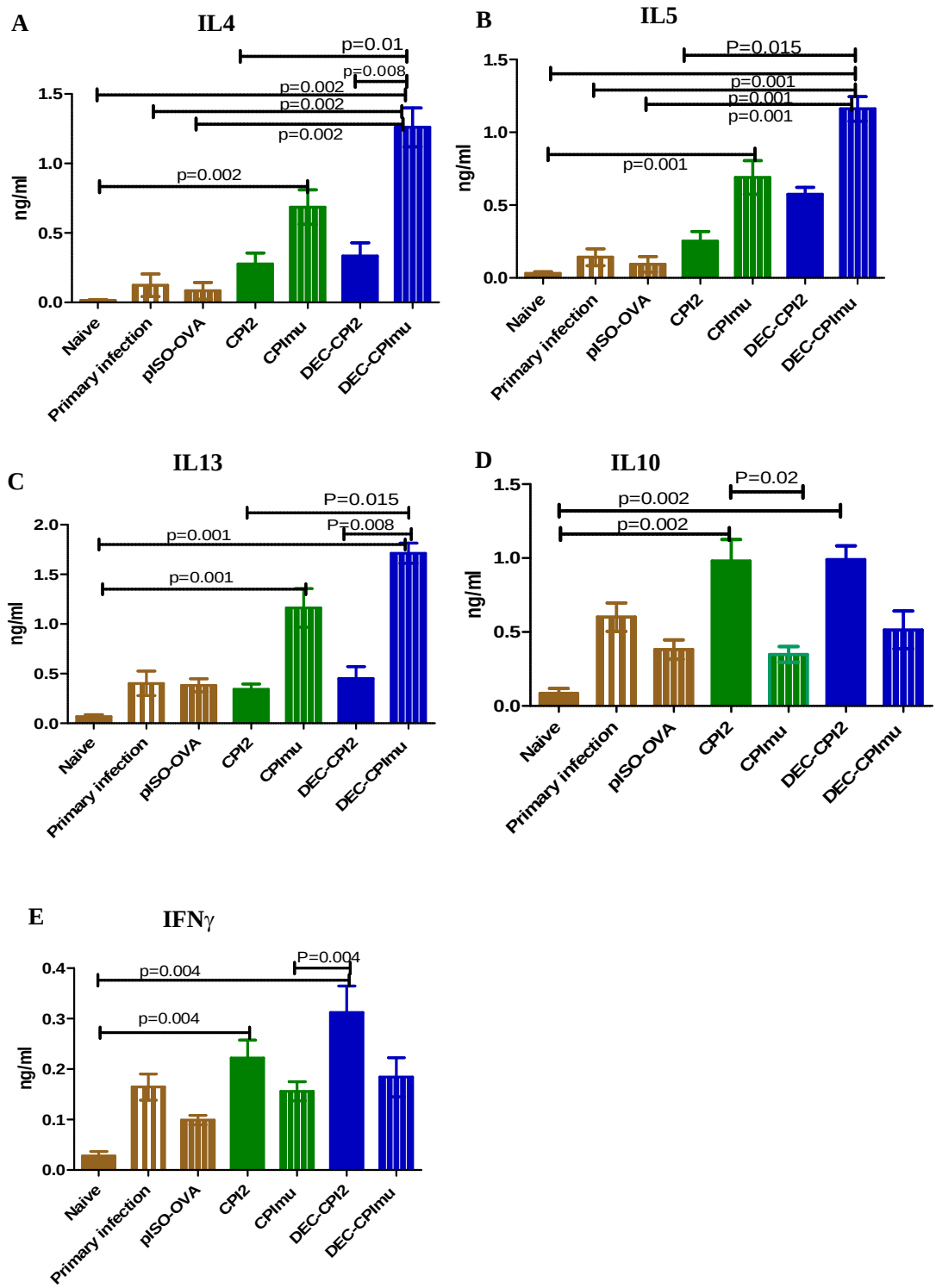


Fig.3.19. Th2-associated cytokines were induced by DEC-CPImu immunization. Supernatant of pleural cavity lavage were harvested and the level of various cytokines (IL4, IL5, IL13, IL10 and IFNγ) were measured by sandwich ELISA- (see methods and materials). Results are shown as the mean of replicate samples. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise. 5 mice per group were used.

3.2.15 CPImu restored the responses induced by CPI2 on the proliferation of lymph node cells *in vitro*

The ability of CPI2 and CPImu, with and without adjuvants (IL4, MIP1α and Flt3L), to stimulate proliferation of T cells was tested using isolated lymph node cells cultured with Ls-Ag and anti-CD3. RPMI 1640 medium was used as a negative control. As presented in Fig. 3.20, the pcDNA3.1-CPI2 could not stimulate the proliferation of lymph node cells after the Ls-Ag-specific stimulation *ex vivo* compared to pcDNA3.1-CPImu which demonstrated an augment of proliferation. Similar impacts were seen in medium and anti-CD3 stimulation. However, the adjuvants IL4, MIP1α and Flt3L only assisted both pcDNA3.1-CPI2 and pcDNA3.1-CPImu to enhance the proliferation with stimulation of medium and anti-CD3, not the Ls-Ag antigen, whereas slightly inhibited the ability of pcDNA3.1-CPImu on cell proliferation. However, no statistically significant difference was found between groups.

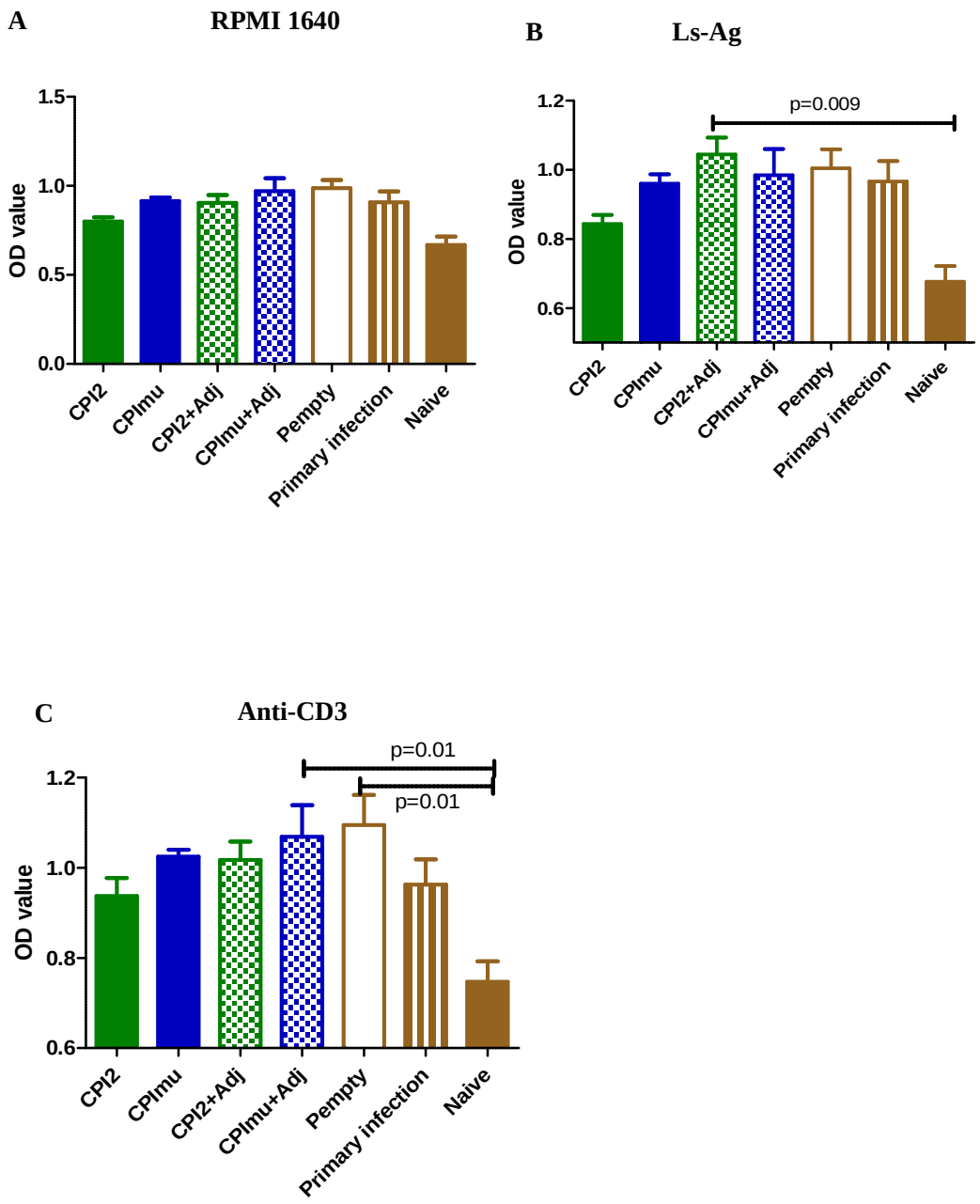


Fig. 3.20 CPImu removed inhibition of CPI2 on the proliferation of lymph node cells *in vitro*. Lymph node cells were cultured in 5% CO_2 incubator at 37℃ for 48 hours followed by the addition of Alamar blue reagent to measure the cell proliferation in 24 hours. Media of RPMI 1640 and Anti-CD3 were set as controls and *L. sigmodontis* excretory/secretory antigen (Ls-Ag) was added to stimulate cells. Adjuvant plasmids encoding IL4, Flt3L and MIP1α were added with CPI2 and CPImu to test their impacts on enhancement of proliferation. 5 mice per group were used. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

3.2.16 Combination of ADDALT and CPImu upregulated the levels of Th2 type antibodies

Although both ADDALT and CPImu candidates could evoke stronger host immune responses, especially when they targeted dendritic cells via the recognition of single chain antibody scDEC205 to the surface receptor DEC205, protection was not achieved. It was therefore decided to test the ability of a combination vaccine of pDEC-ADDALT and pDEC-CPImu to induce protection. The experiment comprised the following: 7 groups were tested including 3 control groups [naïve, primary infection and pcDNA3.1 backbone (pempty)] and pDEC-ADDALT alone, pDEC-CPImu alone and pDEC-ADDALT plus pDEC-CPImu were the test groups. Mice were given 2 doses of immunizations at 2 week intervals simultaneously with adjuvant plasmids encoding IL4, Flt3L and MIP1α. Vaccinated mice were challenged with 30 L3s 4 weeks after the final immunization. Sera (blood), pleural cavity lavage, and lymph nodes were collected after necropsy which was performed 60 days post challenge. Antibody levels were measured by indirect ELISA coated by the *L. sigmodontis* excretory/secretory protein rather than the recombinant ALT and/ or CPI2 protein.

As shown in Fig. 3.21. A, IgG1 levels were increased by the combination immunization compared to the vaccination of pDEC-ADDALT alone, or pDEC-CPImu alone. Thus, antibody titers more than 1×10^7 in log10 were induced by the combined vaccine which was about 10-fold higher than the titers induced by pDEC-ADDALT alone, or pDEC-CPImu alone.

However, the IgG2a level displayed a decreasing trend following immunization with the combination in contrast to the vaccination of pDEC-ADDALT alone and pDEC-CPImu alone. The titers of IgG2a for all groups were lower than IgG1 (Fig.3.21. B). Total IgE levels were also measured. As shown in Fig.3.21. C, the combined vaccination induced greater IgE

than the vaccination of pDEC-ADDALT alone or pDEC-CPImu alone, although there was no significant difference between groups.

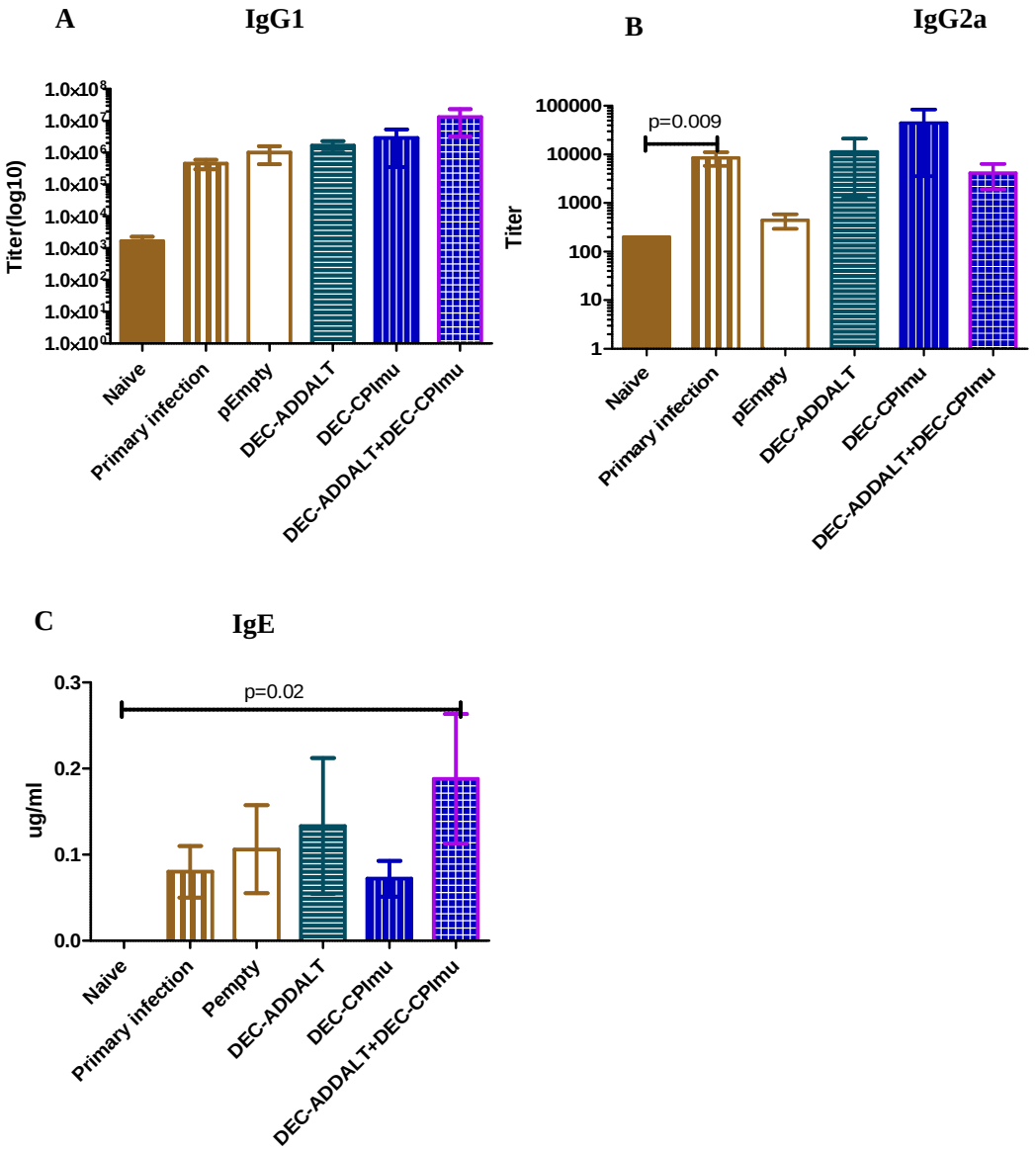


Fig.3.21. Combination of ADDALT and CPImu unregulated the levels of Th2 antibodies. Antibody levels of IgG and IgE were compared among pDEC-ADDALT alone, pDEC-CPImu alone and the combination of pDEC-ADDALT and pDEC-CPImu by indirect ELISA coated with *L. sigmodontis* excretory/secretory protein (see methods and materials). All test groups were co-immunized with the adjuvants IL4, Flt3L and MIP1α. Results are shown as titer of IgG and the mean of replicate samples (+/- S.E.M) for total IgE. 5 mice per group were used. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

3.2.17 IL5 and IL13 levels increased following combined vaccination

The impact of combination vaccination on cytokine production in the pleural cavity and the supernatants of lymph node culture were measured by the capture ELISA. In the pleural cavity, although the level of cytokines remained low, IL5 and IL13 showed a slight increase over control levels. However, the Th1 cytokine IFNγ was never detected either following vaccination with pDEC-ADDALT alone, pDEC-CPImu alone or the combination of pDEC-ADDALT and pDEC-CPImu (Fig.3.22). In lymph node cultures with Ls-Ag, IL13 levels were increased slightly following the combination vaccine compared to immunization with the single vaccines. Other cytokines including IL4, IL5 and IFNγ were undetectable (data not shown). However, no statistically significant difference was found between groups.

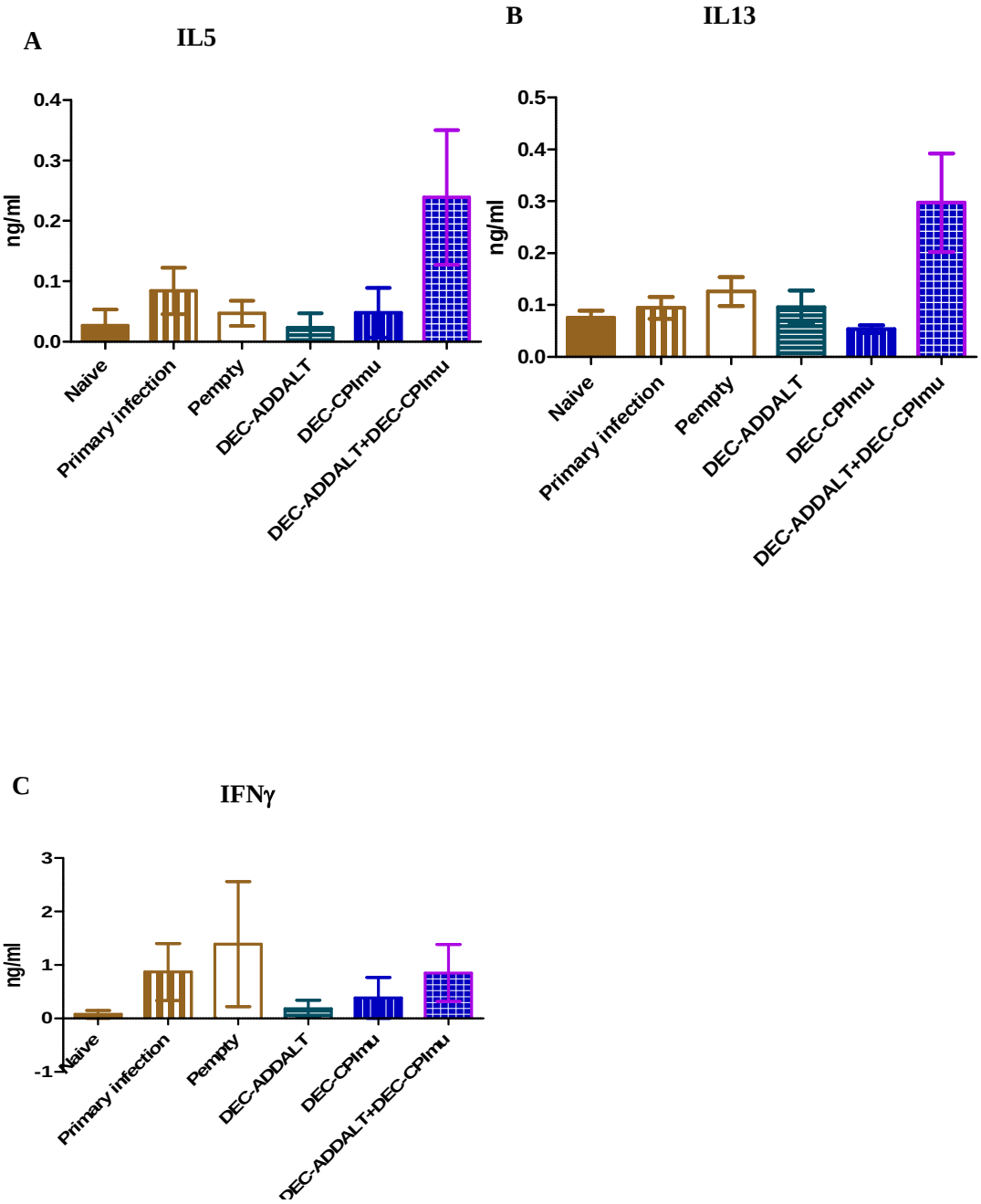


Fig. 3.22 Increased IL5, IL13 were induced by combined vaccination. Supernatant of pleural cavity lavage were harvested and the level of various cytokines (IL5, IL13 and IFNγ) were measured by capture ELISA against the recombinant antigen CPI2 (see methods and materials). Results are shown as the mean of replicate samples. 5 mice per group were used. Mann-Whitney test was used as statistical method. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

3.2.18. Eosinophil numbers increased in the pleural cavity following combined immunization

The cells from the pleural cavity of mice from each of the control and experimental group were concentrated using a cytospin and numbers were counted under a microscope. The pDEC-ADDALT alone, pDEC-CPImu alone and combined vaccinations caused an increase level of total cell recruitment compared to controls, and the combined vaccination induced higher number of cells than pDEC-ADDALT alone, and pDEC-CPImu alone, although no statistically significant difference was found between groups. Regarding eosinophils, a significant increase in combined vaccination was recorded when compared to primary infection and naïve controls. Eosinophil numbers were also raised by immunization with pDEC-ADDALT alone or pDEC-CPImu alone. Numbers of other cell types were also counted, both the macrophages and neutrophils increased in combined vaccination compared to [pempty] control, but the number of macrophages on combined vaccination group was significantly higher than primary infection (Figure 3.23).

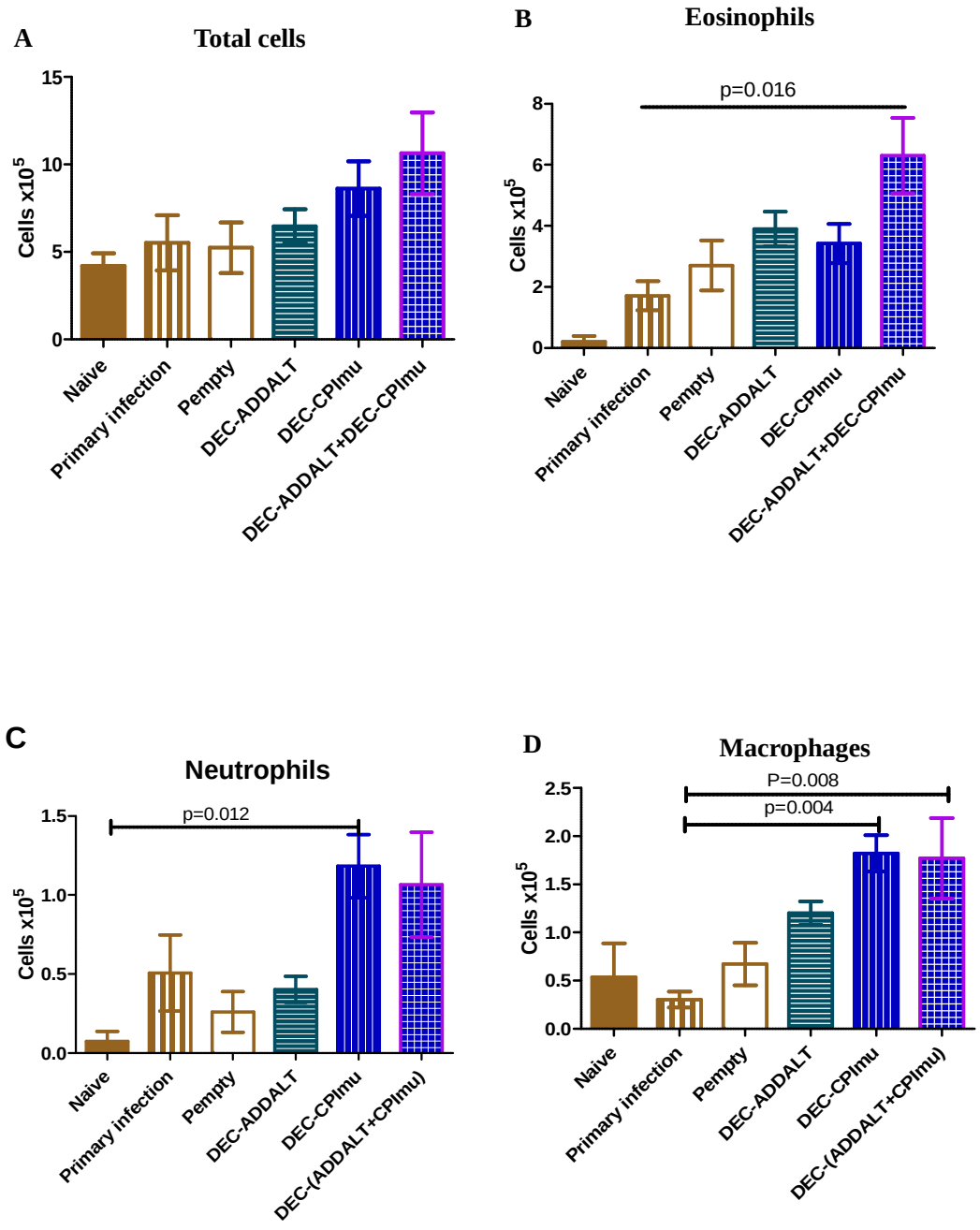


Fig.3.23 Eosinophil numbers in pleural cavity increased following combined immunization. Total cell numbers were determined using the CASY model TT cell counter system. Eosinophil, neutrophils and macrophage numbers were determined following concentration by cytospin and enumerated by microscopy on fixed slides (300 cells at minimum each slide were counted). The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise. 5 mice per group were used.

3.2.19. Significant reduction of worm burden was achieved by combined vaccination

To evaluate the protection elicited by the combined vaccination, adult worms in the pleural cavity and Mf in the blood stream were counted. Mice were challenged with 30 L3s. Protection was recorded as a reduction in worm numbers in test animals compared to those in control groups (Fig.3.24). The mean worm recovery of unvaccinated control mice was 16. The mean recovery of worms from the combined vaccination was 3, which represents about an 82.3% reduction in worm burden. The numbers of worms recovered from mice immunized with pDEC-ADDALT alone or pDEC-CPImu alone were reduced by 38.7% and 50%, respectively. However only the 82.3% reduction observed with the combined vaccine was statistically significant.

As for the number of Mf recovered in the blood, there was an apparent reduction in numbers following vaccination with the pDEC-ADDALT alone, pDEC-CPImu alone or the combined vaccine, and the combined vaccination resulted in a statistically significant reduction of microfilariaemia.

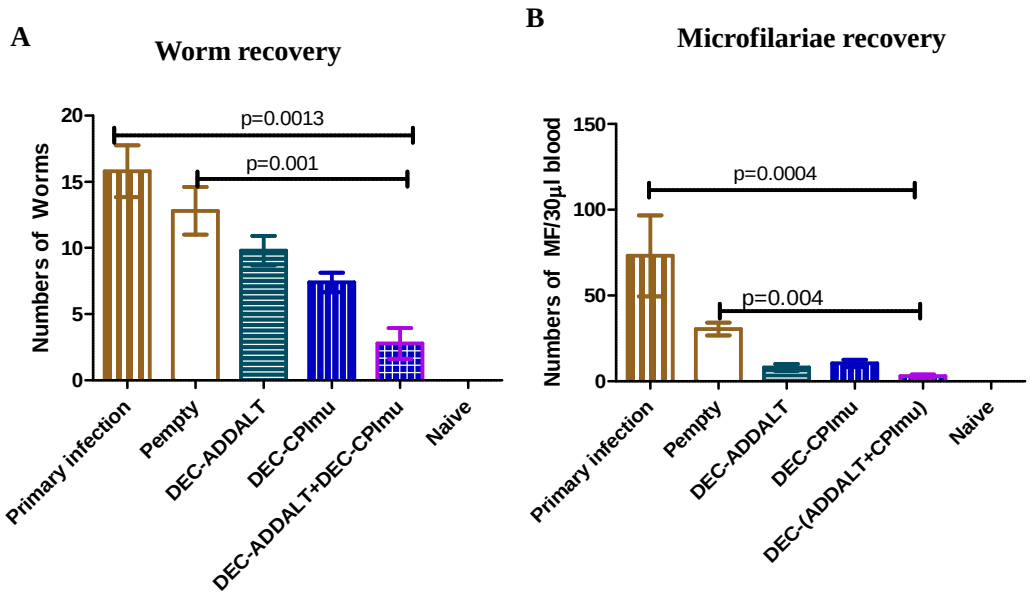


Fig. 3.24. Significant reduction of worm burden was achieved by combined vaccination. Adult worms and microfilariae were counted at 60 day post challenge. Adult worms were recovered in the pleural cavity lavage while microfilaraie were counted in 30 µl blood. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise. 5 mice per group were used.

3.2.20 Immune determinants of protective immunity

In order to identify the main immune determinants of protection, a principal component analysis was performed on all the immunological read-outs that lead to significant reductions of parasite survival (Fig 3.24). 31 variables (Appendix 7) were examined in each mouse and then reduced to 4 principal components that exceeded the explanatory power expected by chance alone, and that represented 51.6% of the variation present in our sampling (Appendix 6, 7; Fig 3.25). Subsequent components were rejected for lack of explanatory power. The first component (PC1) comprised mainly *in vitro* Th2 cytokine responses; PC2 comprised whole worm-specific IgG1, and pleural eosinophils, neutrophils, macrophages but not lymphocytes; PC3 comprised substantial effects of Ls-ALT- and Ls-CPI2-specific IgG1 and IgG2a opposed to equally strong effects of pleural lymphocytes; Finally, PC4 contained mainly pleural cytokines IL5, IL13, IFNγ and pleural lymphocyte numbers. The explanatory

power of the resulting components on parasite survival was assessed by a generalized linear model (GLM). Only the second principal component was significantly correlated with parasite survival ($r = -0.72$, $P < 0.0001$, Fig 3.26). The relative contribution of each antibody class (IgE, IgG1 and IgG2a) to parasite killing was determined. Only IgG1 was found to have a significant effect on parasite killing ($P = 0.026$ with interactions between IgG1 and IgG2a accounted for) whereas IgE and IgG2a had none ($P= 0.7$ and $PGLM = 0.6$, respectively). Likewise, the analysis of respective roles for pleural cell types in protection revealed that parasite killing was attributable to macrophages and neutrophils ($P = 0.02$, $P = 0.003$, and $P = 0.002$), respectively once interactions were accounted for), but not to eosinophils ($P =0.5$)

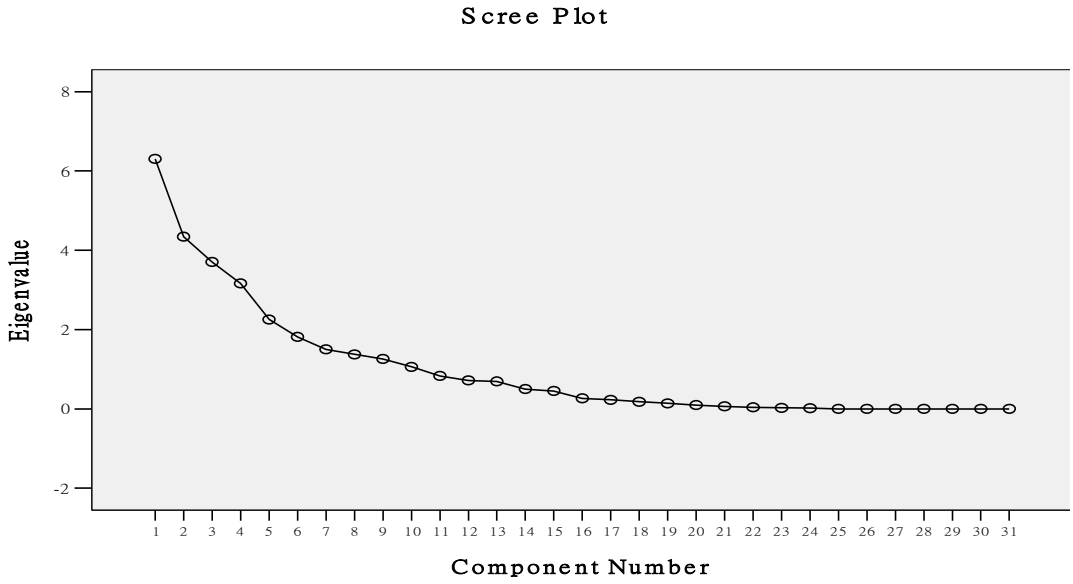


Fig. 3.25. Scree plot of principal component assay. 31 variables (Appendix 7) for each mouse were used in this analysis. The break point was determined to be between 4 and 5 based on the total extraction of the sums of squares loading value (3.163).

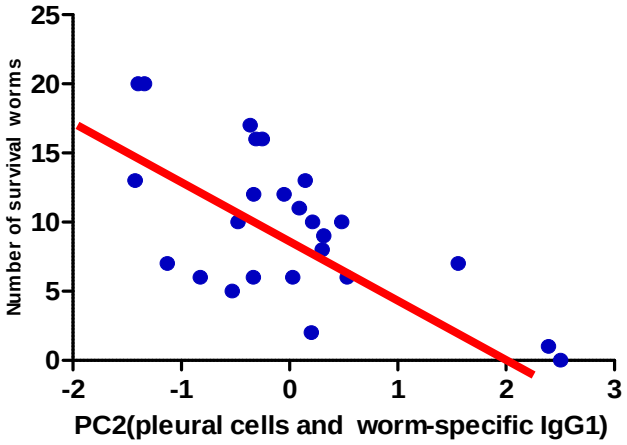


Fig. 3.26. Correlation between worm survival and PC2. Worm numbers recovered and all cells recruited to the pleural cavity (including eosinophils, macrophages, neutrophils but not lymphocytes) and worm specific IgG1 were analyzed. The figure was created by SPSS 13.0.

3.3 Discussion

3.3.1 Ls-ALT and Ls-CPI2 can be expressed in the adult stage of *L. sigmodontis*

The data showed that Ls-ALT-1 and Ls-CPI2 genes had been successfully cloned and identified by enzyme digestion, DNA sequencing, protein expression (in *E. coli* and in COS 7 cell) and Western blotting with sera from mice immunized with Ls-ALT-1/CPI2 DNA vaccines. The ALT gene has been described as L3-specific and highly expressed in *B. malayi* and *W. bancrofti* and it has been suggested that the corresponding protein is involved in survival of filarial nematodes in immunocompetent hosts (Ramachandran S *et al.*, 2004; Thirugnanam S *et al.*, 2007).

In the case of *L. sigmodontis*, the Ls-ALT gene was previously identified from an L3 cDNA

library by using an EST strategy (Allen JE *et al.*, 2000), however, the Ls-ALT-1 gene used in this work was cloned from adult worm mRNA, demonstrating that Ls-ALT-1 is not only expressed in the L3 stage but also the adult stage. This is the same as reported for *O. volvulus* where the ALT gene has been found to be stage-specific (Joseph DT *et al.*, 1998), but can also be expressed restrictly in L2 and L3 stages (Wu Y *et al*, 2004). It should be noted, however, that in this investigation no attempt was made to confirm the presence of the Ls-ALT-1 transcripts in the L3 or L4 stage.

As for Ls-CPI2 gene, the data presented indicated expression of the protein by adult worms which is consistent with the analysis of differential expression of Bm-CPI2 cystatin (Gregory WF *et al.*, 2008), although in this study no attempt was made to determine whether the gene was expressed at all stages. The Ov-CPI2 derived from *O. volvulus* is highly expressed in the cuticle of moulting larvae (Johnstone IL, Barry JD, 1996) but there is no detectable increase in CPI2 transcription around the moulting events in *C. elegans* (Johnstone IL, Barry JD, 1996).

Therefore, Ls-ALT-1 and Ls-CPI2 used in the present experiments were successfully cloned from the adult stage, suggesting that both genes play a role in the parasite-host interaction and may be used as candidates for vaccine development.

3.3.2 Extensive distribution and long time persistence of foreign DNA in host tissue

The distribution of plasmids after immunization is associated with the ability of induction and longevity of immune responses following stimulation by DNA vaccination. To evaluate the gene expression patterns in mice after DNA vaccination, mRNAs from muscle, liver, lung and spleen were extracted and followed by the gene-specific-primer RT-PCR detections. Data presented indicated that Ls-ADDALT was most abundantly expressed in muscle around the tibialis followed by spleen, lung and liver. Ls-CPI2/ CPImu was abundantly expressed in

both muscle and spleen followed by in liver and lung. These data suggest that Ls-ALT/ADDALT and Ls-CPI2/CPImu were extensively distributed in mouse tissue after DNA vaccination. The extensive stimuli of DNA vaccines lead to an interaction between the antigen and the host immune system, thereby inducing subsequent humoral and/or cellular responses. Furthermore, DNA was extracted and detected by PCR using T7/ BGH primers (which indicate the existence of carry plasmids after immunization) instead of specific primers, results showed that all carried plasmids of Ls-ALT/ ADDALT, Ls-CPI2/ CPImu were detectable in muscle, spleen, liver and lung, indicating that genes carried by plasmids were still existed in tissues and might be released to stimulate host immune responses in subsequent time courses. These findings are consistent with the previous reports which have shown that uptake and expression of foreign DNA by muscle cells as well as other tissues such as liver and lung can be maintained after direct injection (Bonifaz LC *et al.*, 2004; Demangel C *et al.*, 2005; Trumpfheller C *et al.*, 2006).

Another important point is the longevity of foreign DNA in mouse tissue. The data presented showed that even 28 days after the final immunization genes were still detectable. Marc D *et al* (2000) using fluorescence-labeled plasmid DNA detected plasmid 24 hours after inoculation in muscle and draining lymph nodes albeit at low level. A comparison with our data suggests present protocols of DNA immunization and delivery have improved persistence of foreign DNA in tissues. This can provide a long term stimulus to the host immune system and consequently lead to the longevity of humoral and cellular responses.

3.3.3 Removal of immunomodulatory properties of Ls-ALT and Ls-CPI2 results in the enhancement of immune responses

The immunomodulatory properties of Ls-ALT-1 and Ls-CPI2 have been mapped according to their amino acid sequences. Bm-ALT-1, which contains one acidic domain (46 aa), is involved in the modulatory function (Maizels RM *et al*., 2008). The corresponding acid domain of *L. sigmondontis* has also been shown to possess similar immunomodulatory

activity (Ls-ALT and Ls-ADDALT based on backbone plasmid pcDNA3.1 had been tested by Simon Babayan, unpublished data). Consequently, DNA plasmids encoding Ls-ALT-1 deleted acidic domain (ADD) were used to immunize BALB/c mice against *L. sigmondontis* L3 challenge. Results showed that the Ls-ADDALT vaccine induced stronger immune responses including IgG1, IgE and Th2 cytokines than Ls-ALT-1without deletion of acidic domain which inhibited host immune responses (data not shown). This observation suggested that the acidic domain prevents Ls-ALT from eliciting protection.

Works presented here have shown that DNA vaccination in BALB/c mice using pcDNA3.1-ADDALT plasmid induced increased IgG1 and IgE antibodies, but not IgG2a, when compared to control plasmids. On the other hand, no predominant cytokine profiles and protection were obtained 10 days after the challenge with 40 L3s. After analysis, it was concluded that the efficiency of DNA uptake and expression might impact the vaccine-mediated immune responses and the 10 days interval between challenge and necropsy might not be long enough to detect resultant cytokine responses. Consequently, a novel DNA vaccine targeting dendritic cells via specific binding to its surface receptor, DEC205, by the single chain Fv monoclonal antibody scDEC205 (encoded by the plasmid vector) was constructed. Immunization with this DEC-ADDALT plasmid increased the IgG2a level and a marginal elevation of IgG1 and IgE. However, immunization with DEC-ADDALT alone did not induce protection.

Analysis of Ls-CPI2 showed it contains the conserved amino acid motifs of QVVAG, PW and SND. The SND motif is responsible for the immunomodulatory properties of Bm-CPI2 (Murray J *et al* (2005). This immunomodulatory property is associated with inhibition of multiple cysteine protease activities found in human B cells and inhibition of the hydrolysis of synthetic substrates. In addition, evidence showed that inhibition of presentation of selected T cell epitopes by antigen-presenting cells existed (Manoury B *et al.,* 2001). These findings provided a good example of a product from a eukaryotic parasite that can directly

interfere with antigen presentation, which may suggest how filarial parasites modulate the host immune response to a helminth invader. More importantly, Murray J *et al* (2005) showed a 10-fold diminished and ablated activity of AEP inhibition, respectively, through site-directed mutagenesis of Bm-CPI2 at Asn-77 (mutated to Asp and Lys).

To remove the AEP inhibition thoroughly, a similar site-directed mutagenesis of Ls-CPI2 gene (substitution of Asn66 with Lys66) was performed. The presented data demonstrated that immunization with Ls-CPI2 DNA vaccine based on pcDNA3.1 backbone inhibited the immune responses with down-regulated IgG1, IgG2a and IgE, and Th2 cytokines IL4, IL5 and IL13, but up-regulated IL10. However, whether the AEP inhibition has been removed thoroughly has not been tested in current study and was not the first concern in this study. T cell proliferation stimulated by the whole *L. sigmondontis* antigens *in vitro* was suppressed by Ls-CPI2. All these findings are generally consistent with the observations of Schierack P *et al* (2003). However, the cystatin used in their experiment induced an up-regulation of nitric oxide production by IFNγ stimulated murine macrophages. Such a response was not seen in the experiments with Ls-CPI2 and Ls-CPImu. Similar results to Schierack P *et al* (2003) have been reported by Hartmann S *et al* (1997). However, all these immunosuppressions caused by Ls-CPI2 were reversed by vaccination using its mutant Ls-CPImu with significant enhancement, which proved that the strategy of removal of immunosuppressive motifs in Ls-CPI2 can circumvent the parasite immunomodulation and thereby enhance the host immune responses.

Both Ls-ALT-1 and Ls-CPI2 have the immunomodulatory properties that may play a crucial role on the establishment and persistence of filarial infection. However, these immunomodulatory characteristics have been removed from modified sequences and both Ls-ADDALT and Ls-CPImu have been shown to evoke Th2 immune responses which can kill the parasites.

3.3.4 DCs are essential to the enhancement of DNA vaccination

Besides the circumvention of parasite immunomodulation strategy, it was presumed that the effects of vaccination may depend on DNA antigen uptake efficiency. A fundamental initial step in vaccination is that the proteins need to be taken up, processed, and presented by DCs. Following intradermal injection of a plasmid DNA vaccine into mice, the encoded gene is expressed in transfected keratinocytes and myocytes at the site of inoculation (Wolff JA *et al.*, 1990) as well as a small number of DCs (Akbari O *et al.*, 1999a; Bot A *et al.*, 2000). Keratinocytes and myocytes lack MHC II and co-stimulatory molecules, which renders them poorly effective at presenting antigen and priming naïve immune cells (Wiendl H *et al.*, 2005), and they do not have ready access to T cells in lymphoid tissues, as is the case for DCs (Granelli-Piperno A *et al.*, 2005; Lindquist RL *et al.*, 2004). Therefore, it is thought that immune priming begins a few transduced DCs then boosts immunity (Cho JH *et al.*, 2001; Corr M *et al*., 1999). Consequently, uptake of DNA vaccines could be enhanced by directly targeting the encoded protein to DCs.

Works by other researchers demonstrated that antigens can be targeted selectively to DCs *in vivo* when they are fused into antibody against DEC205 (Hawiger D *et al.*, 2001), this interaction can lead to high efficient antigen processing and presentation on MHC I and II products and strong protective T cell immunity (Bonifaz LC *et al.*, 2004; Trumpfheller C *et al.*, 2006). Therefore, plasmids based on the scDEC205 backbone plasmids (which were kindly made available by Dr. Ralph Steinmann) were constructed to enhance DNA vaccine-induced immune responses by targeting DCs. Results showed that immunization with DEC-ADDALT plasmid induced stronger IgG2a, IgG1 and IgE immune responses compared with pcDNA-ADDALT vaccination which was not targeting DCs directly in BALB/c mice. Significant increases were seen with DEC-CPImu vaccination. In contrast to the low level of immune responses elicited by pcDNA-CPImu, statistically significant increases in IgG1, IgG2a and IgE as well as Th2 cytokines including IL4, IL5 and IL13 were produced by DEC-CPImu immunization, suggesting targeting DCs via its surface receptor

DEC205 enhanced the efficacy of DNA vaccination. This is consistent with the experiment reported by Demangel C *et al* (2005) on the capacity of a single-chain antibody, anti-DEC205. Results showed targeting DNA vaccine antigens in mice resulted in a roughly 2-fold increase in B and T cell responses to a mycobacterial antigen. Furthermore, these findings are supported by Nchinda G *et al* (2008), they generated a DNA vaccine encoding a fusion protein HIV gag p41 and a single-chain Fv antibody (scFv) which was specific for the DC-restricted antigen-uptake receptor DEC205. Results showed immune responses were enhanced greatly by DEC205 targeting.

3.3.5 Th2-bias induced by Ls-ADDALT and Ls-CPImu DNA vaccinations

The presented data showed that, when DNA vaccinations were performed in BALB/c mice, Ls-ADDALT and Ls-CPImu DNA vaccinations significantly enhanced the levels of IgG1, IgE, IL4, IL5, and IL13 and significantly decreased the level of IFNγ when compared to the immunization with CPI2 (presented as pcDNACPI2 or DEC-CPI2). These findings are similar in profile to the protective immunity induced by the irradiated L3 immunization in mice, cats and dogs (Jian X *et al.*, 2006; Le Goff L *et al.*, 1997; Le Goff L *et al.*, 2000; Babayan SA *et al.*, 2006; Ricardo T, 2006). However, some differences between presented data and vaccinations of irradiated L3s were found. First, both Ls-ADD ALT and Ls-CPImu DNA vaccinations mainly induced IgG1 antibody not both IgG1 and IgG2 or IgM (IgM was not measured in present studies). Second, the cytokine IL13 was elevated by DNA vaccinations in present studies and seldom detected following irradiated L3 vaccination.

Though differences exist between present findings and irradiated L3 vaccinations, one agreement has been reached that vaccination success depends on Th2-biased immune responses especially IL5, IL4, IgE and IgG1 and /or eosinophils (Martin C *et al.*, 2000; Martin C *et al.*, 2001; Abraham D *et al.*, 2004).

3.3.6 IL10 produced by DNA vaccine did not induce inhibition of Th2-mediated protective immunity

Production of IL10 has been reported to be associated with the downregulatory pathway. IL10 produced by $CD4^+CD25^+$ Tregs is associated with the inhibition of Th1 and Th2 response (Hesse M *et al.*, 2004; McKee AS, Pearce EJ, 2004). Similarly, IL10 deficiency has been found to enhance the Th1-mediated killing of muscle larvae of *T. spiralis* (Helmby H, Grencis RK., 2003). Although IL10 might play a role on regulation of *L. sigmodontis* infection, Taylor MD *et al* (2005) believed that it was not the key role or sole mechanism for $CD4^+CD25^+$ Treg action in light of the fact that neutralization of IL10R did not restore T cell responsiveness *in vitro* and failed to restore protective immunity *in vivo*. However, IL10 is required to promote Th2 responses and it has been shown that IL10 deficient mice fail to induce protective Th2 responses against *Trichuris muris* (Schopf *et al.*, 2002).

As for presented data, Ls-CPI2 vaccination induced elevated IL10 toward L3 challenge, which is consistent with the report that CPI proteins elicited an IL10 response against *A.viteae* (Hartmann S *et al.*, 1997). However, IL10 production by Ls-CPImu vaccinated mice decreased significantly following L3 challenge and was undetectable after dual vaccinations with Ls-ADDALT and Ls-CPImu. Nevertheless, a cocktail of Ls-CPImu and Ls-ADDALT did induce a protective immunity. It is possible that the induction and regulation role of IL10 are performed in a dose dependent manner, with low levels promoting Th2 and protection and high levels drive regulation.

3.3.7 Worm specific IgG1 and neutrophils, macrophages are correlated with worm killing

Present data showed that neither DEC-ADDALT alone nor DEC-CPImu alone could induce protection while dual vaccination (combination of DEC-ADDALT and DEC-CPImu)

significantly reduced the number of worms (Fig. 3.24). This protective immunity appears to be associated with worm specific IgG1, neutrophils and macrophages.

The mechanism of parasite killing is not fully understood. Classically, it is associated with the antibody-dependent cell-mediated cytotoxicity (ADCC, Chandrashekar R *et al*, 1990), in which a large amount of Th2 type antibodies are involved including IgG1 and IgE. The present data showed high levels of IgG1 following combination vaccination, suggesting it plays a protective role. However, as mentioned previous, IgG2a might be responsible to the adult worm killing based on the fact that Th1 and Th2 responses might synergize to donate to protective responses. According to present findings, it appears to be more biased toward IgG1 rather than IgG2a, and this might be because the vaccine design strategy in the present study was mainly aimed at enhancing Th2 rather than Th1 responses; However, it does not mean the IgG2a does not contribute to the protection in general.

The present study did not determine whether antibody is involved in Mf killing. Earlier studies showed that the μMT mice prolonged survival of *B. malayi* Mf which is due to a detection of antibody, not the lack of B cells (Gray CA, Lawrence RA, 2002). Mf killing in this study might be the consequence of adult worm killing, which may be expected to reduce the number of circulating Mf. A reduced Mf would be expected to reduce morbidity and severe disease. However, in most lymphatic filariasis, where Mf is in the blood, the disease is associated with the adult worms. So again, killing Mf instead of adult worms is not the main target to design vaccines.

Besides IgG1, in the present study, cells including macrophages and neutrophils recruited into the pleural cavity contributed to worm killing. Macrophages are not only innate immune cells in the primary response to filarial infection, but also play a role on coordination of the adaptive immune response, inflammation, resolution and tissue repairs (Delavary BM *et al*, 2011). The classic macrophage activation is required to kill intracellular pathogen while alternatively activated macrophages driven by IL4 and IL13 are a different phenotype that is

important for the immune response to parasites (Gordon S, Martinez FO, 2010). Combination vaccine immunization in the present study induced increased IL13, which may trigger the macrophage activation and worm killing. This activation may be dependent on the up-regulation of arginase, Fizz-1 and Ym-1. However, the phenotypes of macrophages recruited to the pleural cavity 60 days post challenge were not investigated because the aim of present experiment was to measure the effect not the causes of vaccination. It was assumed that both types of macrophage might be involved in the worm killing but dominant one might be alternatively activated macrophage which are however to be part of Th2 responses on parasite killings (Gordon S, Martinez FO, 2010).

Neutrophils are reported to be the first cells recruited in response to a helminth infection, and have been suggested to play a protective role during *L. sigmodontis* infection (Saeftel M *et al.*, 2001; Saeftel M *et al.*, 2003). In the present findings, neutrophils increased in the pleural cavity 60 days post infection when worms develop to the adult stage and live in the pleural cavity. The increased neutrophils may kill worms by forming nodules around the worms. In addition, the increased IL5 might enhance worm killing as well as the IgG1 antibody, which was also described to play a crucial role in *Strongyloides* sp worm killing by cooperation with neutrophils through ADCC mechanisms (Brigandi RA *et al.*, 1996).

However, eosinophils, which are well-documented to be effector cells in human helminth infection, did not appear to correlate with worm killing in the present study. This might relate to the eosinophil activation state, not the number of cells, and the infection stage. As documented, eosinophils are capable of degranulation and selective release of granule proteins in a process mediated by eosinophil vesicles (Melo RCN *et al.*, 2008; Walsh GM, 2001). Degranulation is triggered by FcRs recognizing antibody-bound antigen. Several cytokines including IL3, IL5, granulocyte macrophage colony stimulating factor (GM-CSF), TNF, IFNβ, and platelet activating factor (PAF) can enhance or directly trigger this process. Human eosinophils express FccRI, FccRIIα, FccRIIβ, FccRIII, FcεRII and FcαR, however, mouse eosinophils do not express the high-affinity IgE FcεRI, and therefore do not

deregulate as readily. Thus, despite of significantly elevated eosinophils in the present combination vaccination, effective activation might not be triggered and consequently the cells may not be involved in worm killing in this system.

Chapter 4-Immune responses against filarial infection were enhanced by single VAH or TPX vaccination

4.1 Introduction

Vaccination with antigens derived from iL3 are of particular interest, because L3s are important for the establishment of infection and represent key targets of protective immunity (Gregory WF *et al.*, 2000). It has been proposed that antigens secreted from L3 that are recognized by putative immune individuals may make suitable targets for vaccine development. One such antigen, VAH, a homologue of insect venom allergen identified earlier from *B. malayi*, had been shown to be a promising vaccine candidate in experimental animal models (Murray J *et al.*, 2001; MacDonald AJ *et al.*, 2004). One study also suggested that VAH is a potent adjuvant for other antigens (MacDonald AJ *et al.*, 2004).

An alternative rationale is to target molecules that are known to assist the parasite in its long survival in an immuno-competent host. One such molecule is the thioredoxin peroxidase (TPX) that is highly expressed by all filarial life cycle stages. TPXs are a new class of antioxidant molecules identified from nematode *B. malayi*, although the family was previously described from yeast (Kim IH *et al.*, 1989; Kim K *et al.*, 1988). Works on the yeast TPX showed that this antioxidant is capable of reducing H_2O_2 and alkyl hydroperoxides (Chae H *et al.*, 1994). It is known that cytotoxic molecules such as oxygen radicals and nitric oxide released from the immune system can damage filariae. It has become clear that helminths do produce enzymes with antioxidant capabilities, With example of both extracellular and cytoplasmic forms of Cu/ Zn superoxide dismutase (SOD) (Henkle KJ *et al.*, 1991; Hong Z *et al.*, 1991; James ER *et al.*, 1994; Rhoads ML, 1983; Tang L *et al.*, 1994). Moreover, a glutathione peroxidase has been shown to be one of the major surface-associated molecules on filarial nematodes (Cookson E *et al.*, 1992). Such considerations identify TPX as a potential vaccine candidate.

The works presented below focus on immune responses to the *L. sigmodontis* genes, Ls-VAH and Ls-TPX, and their use as DNA vaccines. Again, the potential of targeting DEC205 on the surface of DCs and thereby enhance immunogenicity was tested.

4.2 Results

4.2.1 Isolation of Ls-VAH and Ls-TPX genes

VAH is a member of venom allergen like protein (VAL) family related to the *Ancylostoma* secreted protein (ASP) antigen, and has been identified to be a protective vaccine candidate and be associated with angiogenesis. The VAH gene sequence derived from *L. sigmodontis* was downloaded from Genebank (http://www.ncbi.nlm.nih.gov/). The full ORF (504 bp) was isolated from adult worms and cloned into the pcDNA3.1 expression vector (Fig. 4.1 A) and sequenced and analyzed by the BLASTn and BLASTp programs at the NCBI website. Results showed the isolated gene shares 82% identity with *W. bancrofti* vespid allergen antigen homolog (AF109794) at the gene level and with 84% identity at protein level with venom allergen antigen-like protein 1 (XP-001894273). Alignments with *W. bancrofti* VAH-1(AF109794), *D. immitis* VAL (AF001100) and *O. volvulus* ASP-1 (AF020586) revealed that the Ls-VAH gene possesses almost all the conserved domains except the N-glycosylation site (Fig. 4.3).

The Ls-TPX gene sequence recorded in Genebank contains an ORF of 645 bp, however, the gene cloned for this work contained a 723bp ORF (Fig. 4.1.B). An alignment of Ls-TPX with members of TPX/ TSA family described from *B. malayi* (Bm-TPX-1, -2; U34251, U47100), *D. immitis* (Di-TPX; AF001007), *F. hepatica* (Fu-TPX; U88577), *Mus musculus Mer* 5 (Mu-TPX; M28723), *O. volvulus* (Ov-TPX; R95400), *Saccharomyces cerevisiae* (Sc-TPX; L14640) is presented in Fig. 4.2. The overall level of identity and similarity between Ls-TPX and the other TPX proteins ranged from 45% to 90% (Fig. 4.2. A, B). The amino acid sequence of Ls-TPX showed a high identity of 89.4% to Bm-TPX1. For all the

members of TPX/ TSA family proteins, there is a high degree of sequence identity upstream and downstream from the conserved amino-terminal Cys with the consensus of Motif 1. The level of sequence conservation surrounding the carboxy-terminal Cys was notably lower (consensus of motif 2).

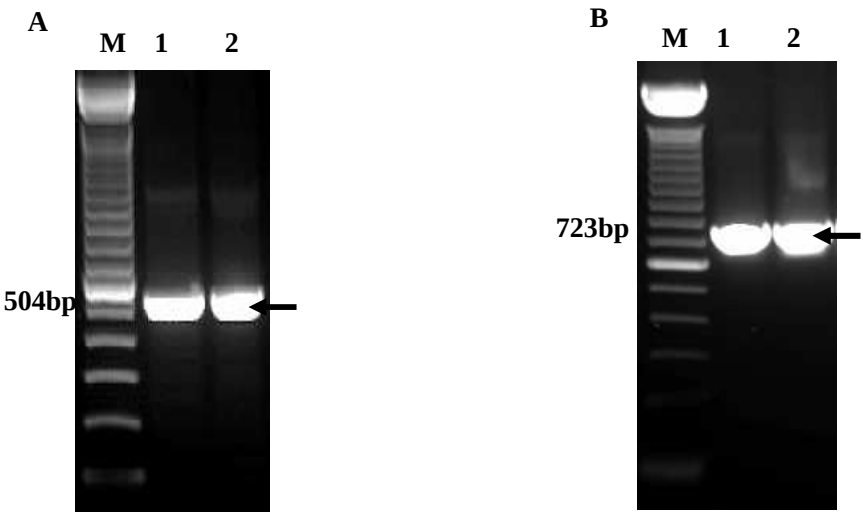
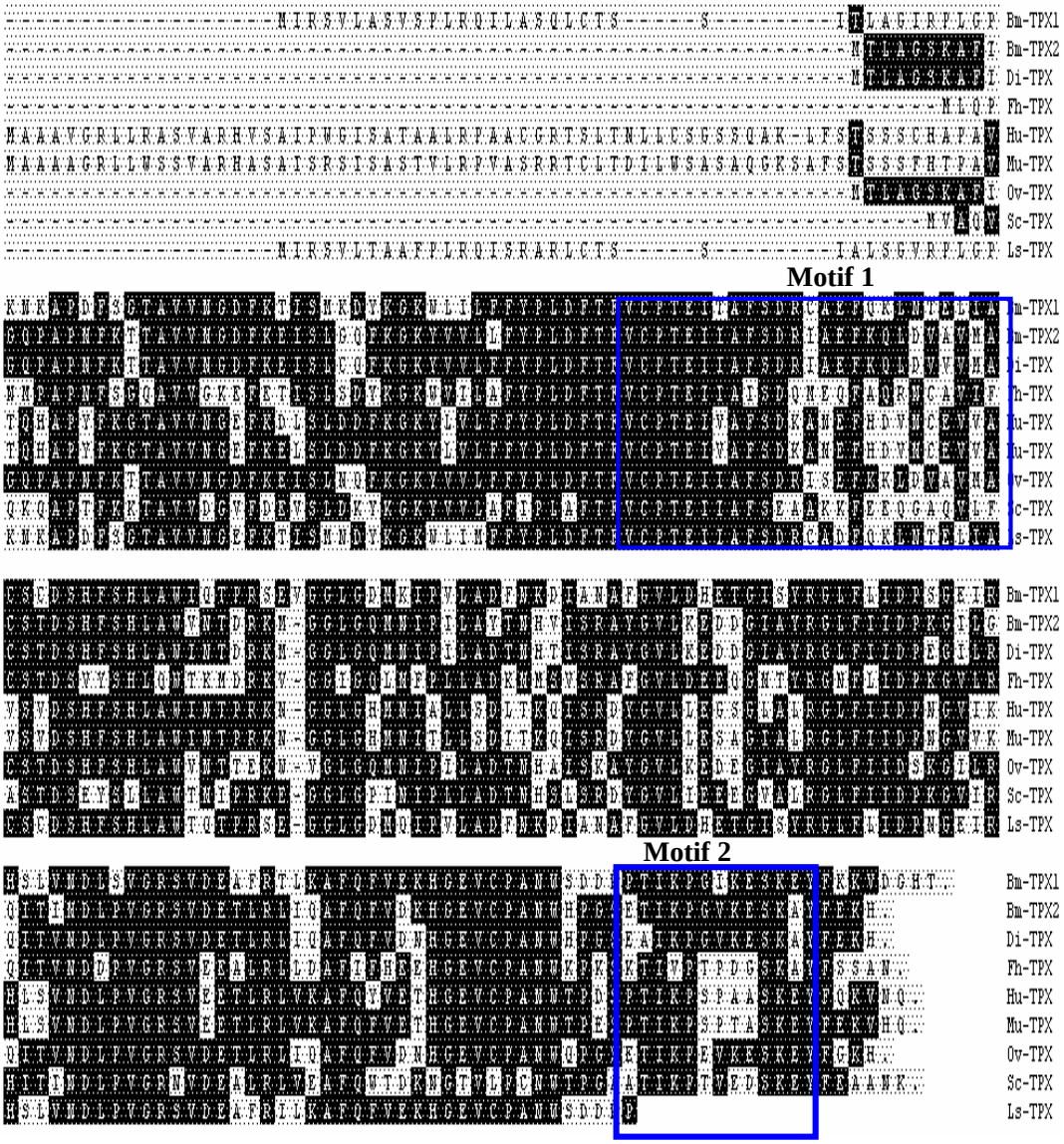


Fig. 4.1 Agarose gel electrophoresis of PCR amplification of VAH and TPX in 0.5% TBE at 120 V for 40 minutes. PCR was performed to amplify the full ORF of VAH and TPX. mRNAs derived from adult worms were used as the templates. (A) PCR products of VAH. M. 100 bp marker; 1, 2. VAH; (B). PCR products of TPX. M. 100 bp marker; 1, 2. TPX.

A



B

Percent Identity

	1	2	3	4	5	6	7	8	9		
1		59.5	61.0	56.4	52.2	89.4	55.7	60.3	48.2	**1**	Bm-TPX1
2	57.6		94.0	59.0	61.5	53.0	63.5	89.9	59.9	**2**	Bm-TPX2
3	54.5	5.7		59.0	63.0	54.5	65.0	90.5	58.9	**3**	Di-TPX
4	65.4	58.1	58.1		53.8	53.8	53.8	56.9	54.9	**4**	Fh-TPX
5	68.3	53.6	50.6	70.1		52.4	85.6	61.8	57.9	**5**	Hu-TPX
6	10.9	60.9	57.5	58.0	66.8		56.2	53.8	45.2	**6**	Ls-TPX
7	67.3	49.7	46.9	70.1	15.1	65.7		63.3	57.9	**7**	Mu-TPX
8	57.0	10.8	10.2	61.8	52.9	59.7	50.0		58.4	**8**	Ov-TPX2
9	83.3	54.5	56.6	67.7	59.9	79.9	59.9	57.0		**9**	Sc-TPX
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**		

Divergence

C

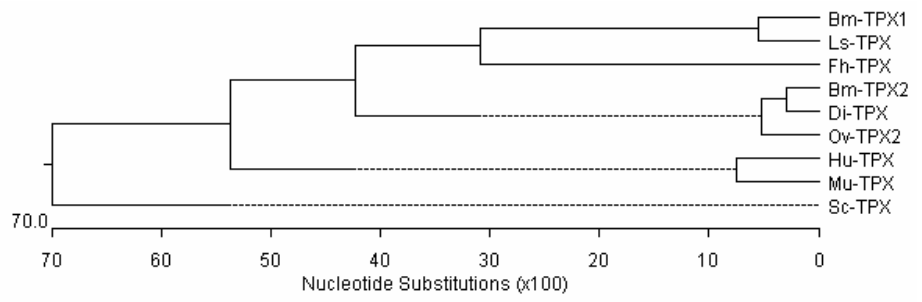
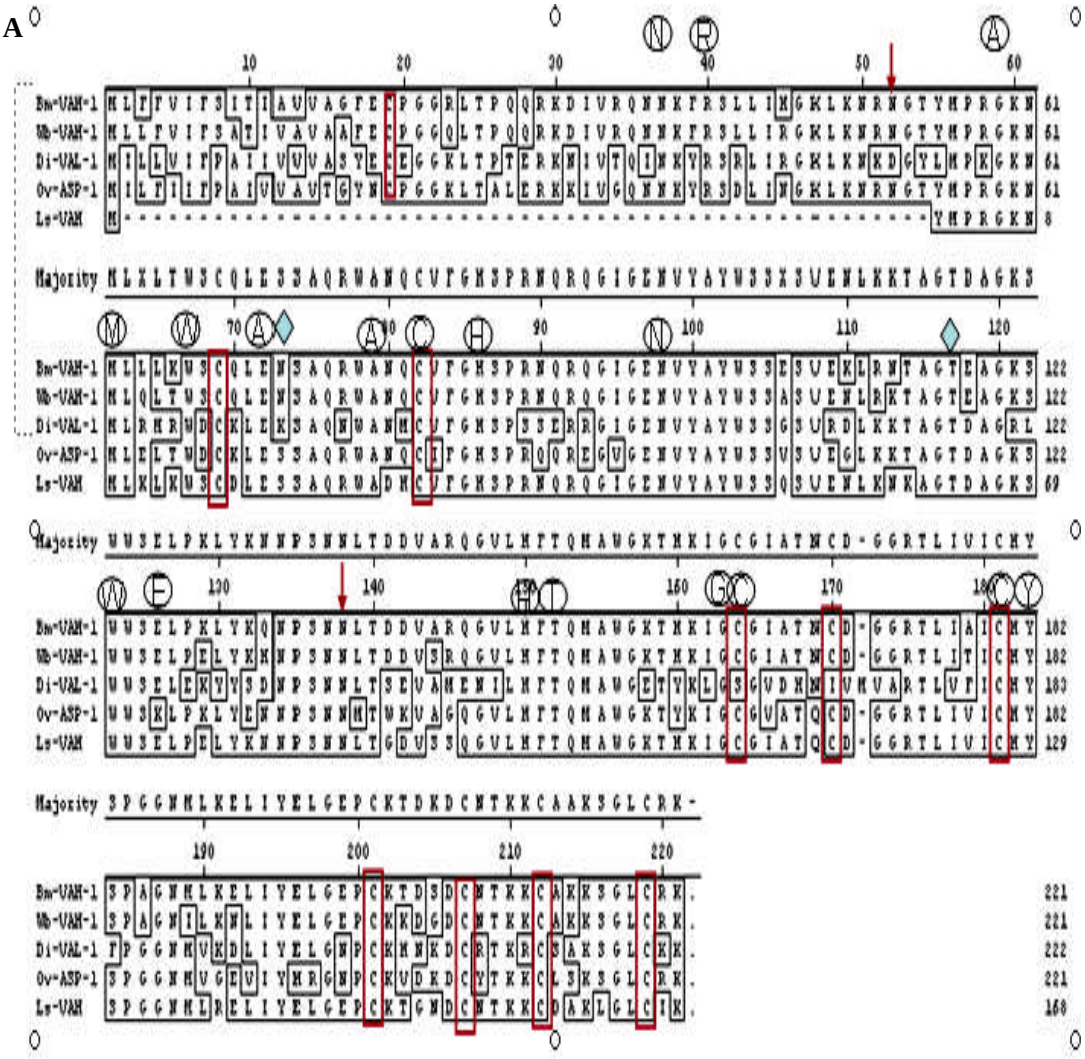


Fig.4.2. Comparison of *L. sigmodontis* TPX and other TPX sequences . (A).Alignment of the deduced amino acid sequence of *L. sigmodontis* Ls-TPX with TPX/TSA proteins. Alignment of *L. sigmodontis* Ls-TPX with TPX/TSA proteins reported from *B.malayi* (Bm-TPX-1 U34251; Bm-TPX-2 U47100), *D.immitis* (Di-TPX; AF001007), *F.hepatica* (Fu-TPX; U88577), *Mus musculus* Mer 5 (Mu-TPX; M28723), *O.volvulus* (Ov-TPX2; R95400), *S. cerevisiae* (Sc-TPX; L14640) using Clustal W method. Regions shaded in black designate residues identical to Ls-TPX. The conserved TPX motifs were boxed and labled. (B). Outline of the percent identity and divergence between the TPX proteins based on the alignment outcomes. (C). Outline of the phylogenetic trees of the TPX proteins based on the alignment outcomes.

A



B **Percent Identity**

	1	2	3	4	5	6	7	8	
1		30.3	32.4	30.4	30.3	29.9	26.4	34.5	1
2	147.6		62.9	24.4	73.3	90.5	25.3	83.9	2
3	133.1	49.1		25.2	66.1	63.8	23.0	62.5	3
4	140.9	184.1	173.3		29.0	23.5	21.3	29.8	4
5	142.5	32.3	44.2	166.7		73.8	23.5	76.2	5
6	153.0	9.7	48.3	184.1	31.6		25.3	82.7	6
7	166.0	158.3	189.2	221.0	172.1	158.3		25.6	7
8	120.5	17.4	50.5	165.2	27.8	18.9	149.8		8
	1	2	3	4	5	6	7	8	

Divergence

Ac-ASP-1
Bm-VAH-1
Di-VAL-1
Hc24
Ov-ASP-1
Wb-VAH-1
SOLI 3
Ls-VAH

C

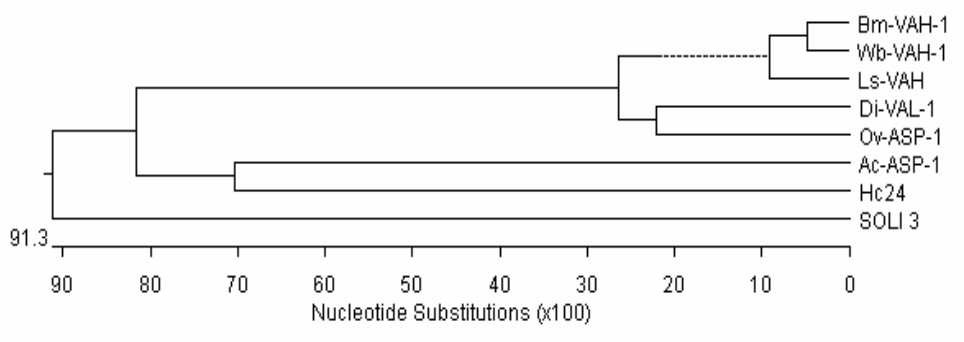


Fig. 4.3. Comparison of *L. sigmodontis* VAH and other VAH sequences . (A). Alignment of *L. sigmodontis* VAH proteins with *B. malayi, W. bancrofti, O. volvulus* and *D. immitis*. Alignments were prepared using the ClustalW facility on Lasergene Suite 5.0. All cysteine residues are displayed in red. Arrows identify the two potential *N*-glycosylation sites, one of which is shared with *W. bancrofti*, one of which is defect. Rhombus denote condos found to show synonymous nucleotide polymorphisms (AAC/AAT, both Ask; and ACC/ACG, both Thr), red rectangle denoting conservation within the nematode sequences shown. Accession numbers of the sequences presented are as follows: *W. bancrofti* VAH-1, AF109794; *D. immitis* VAL, AF001100; *O.* v*olvulus* ASP-1, AF020586; Hc24 (derives from *H. contortus*), AAC03562; *A. Caninum,* AAD31839; *L. sigmodontis* VAH. (B) Sequence distance result of alignment of VAH proteins. Clustal W in MegAlign program was used to construct alignment. *W. bancrofti* VAH-1, AF109794; *D. immitis* VAL, AF001100; *O.* v*olulus* ASP-1, AF020586; Hc24, AAC03562; *A. caninum*, AAD31839; *L. sigmodontis* VAH were applied to process; *Solonepsis invicta* (fire ant) allergen, SOLI3, AF012919. (C). Outline of the phylogenetic trees of the VAH proteins based on the alignment outcomes.

4.2.2 Construction of plasmids

PCR products of VAH and TPX for pcDNA3.1- constructs were ligated into pcDNA^{TM}3.1 Directional TOPO expression vector according to the kit instructions. The pDEC205-plasmids contain the single chain antibody (scDEC205), which targets to the DEC205 receptor on the surface of DCs, or a single chained antibody with irrelevant specificity (sc-ISO) as the control. Before the ligation into vectors, pDEC205-OVA and pISO-OVA were double digested with NotI/ XbaI enzymes (Figure.3.5 A). Then, NotI/ XbaI-digested PCR products of VAH and TPX were ligated into digested pDEC205- and pISO- to replace the OVA gene on the vector, respectively. Clones were confirmed by PCR using T7 and BGH primers, respectively (Fig. 4.4). Expression plasmids of pET24a-VAH and pET29c-TPX

were constructed by the ligation of the NdeI/XhoI digested PCR product of VAH and TPX with NdeI/ XhoI digested pET24a and pET29c, respectively. Clones were confirmed by PCR using T7 and T7 terminator primers (Fig. 4.5). The plasmids of pcDNA3.1-VAH, pDEC205-VAH, pcDNA3.1-TPX, pDEC205-TPX, pISO-VAH, pISO-TPX, pET24a-VAH and pET29C-TPX were sequenced and results showed all plasmids were successfully constructed.

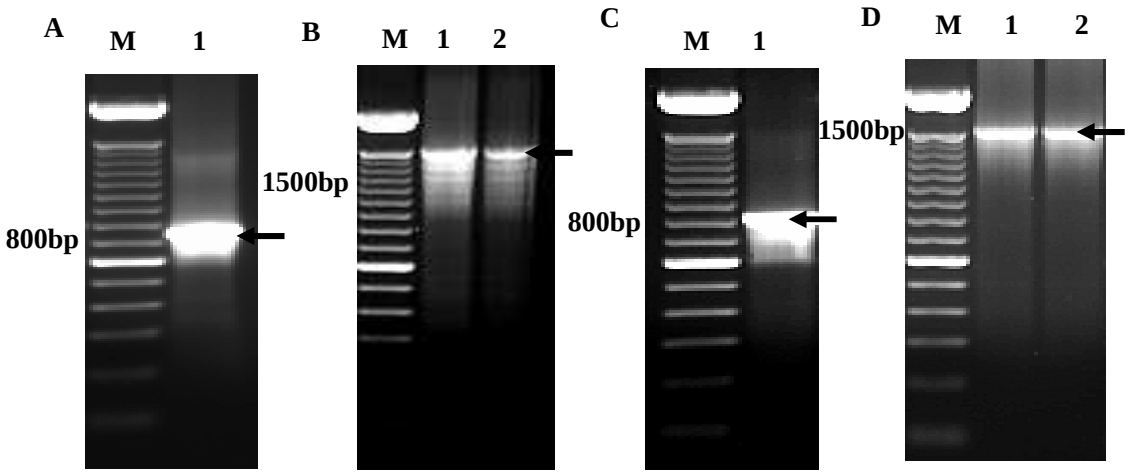


Fig. 4.4 Agarose gel electrophoresis of PCR identification of plasmids in 0.5% TBE at 120 V for 40 minutes. (A).pcDNA3.1-VAH using T7/BGH primers. M, 100bp marker; 1, pcDNA3.1-VAH; (B) pDEC205/ pISO-VAH using T7/ BGH primers. M, 100bp marker. 1, pDEC205-VAH. 2, pISO-VAH. (C) pcDNA3.1-TPX using T7/ BGH primers. M, 100bp marker; 1, pcDNA3.1-TPX; (D) pDEC205-TPX and pISO-TPX using T7/ BGH primers . M, 100bp marker. 1, pDEC205-TPX ,2, pISO-TPX;

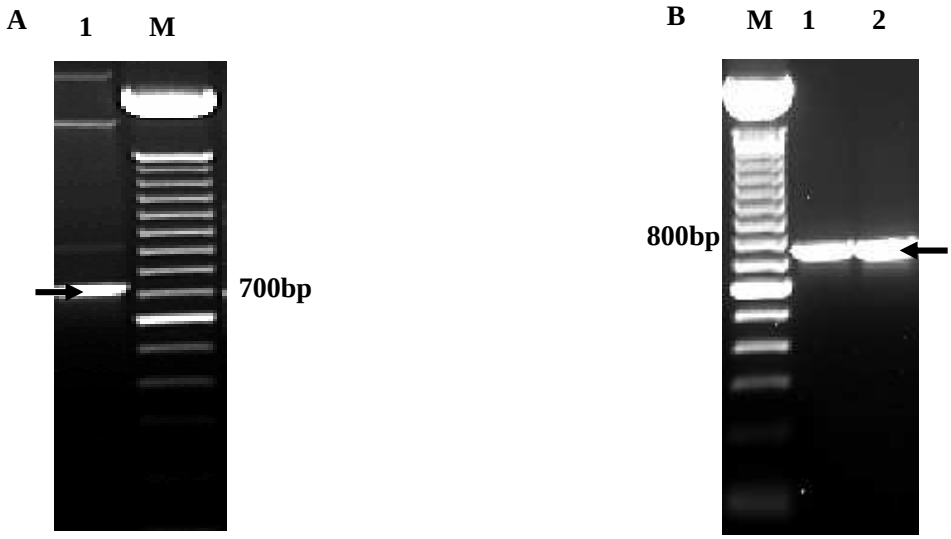


Fig. 4.5 Agarose gel electrophoresis of PCR identification of expression plasmids in 0.5% TBE at 120 V for 40 minutes. (A) pET24a-VAH using T7/ T7 terminator primers. M, 100bp marker. 1, pET24a-VAH; (B) pET29c-TPX using T7/ T7 terminator primers. M, 100bp marker. 1, pET29c-TPX.

4.2.3 Protein expression in *E. coli* and purification

To prepare antigens for ELISA, VAH and TPX genes were sub-cloned into the expression vector pET24a and pET29c, respectively which produce recombinant protein containing a poly His tag. To determine the optimal expression conditions, IPTG concentration (0.5 mM,1 mM, 2 mM), host strain (competent cell BL21-DE3 and Rosseta-gami 2), and temperature (22°C, 25°C, 30°C, 37°C) were tested. Pellets and supernatant fluids were collected at 1, 2, 3, and 4 hours after IPTG induction and assayed by SDS-PAGE electrophoresis. As shown in Figure 4.6 the protein of VAH was expressed best at 37°C 3 hour after 0.5mM IPTG induction while TPX was expressed best at 37°C 4 hour after 1 mM IPTG induction and the pellet contained most of both of the expressed proteins. The concentration of purified protein detected by Coomassie (Bradford) Protein Assay was 1.26 mg/ml for VAH and 0.47 mg/ml for TPX.

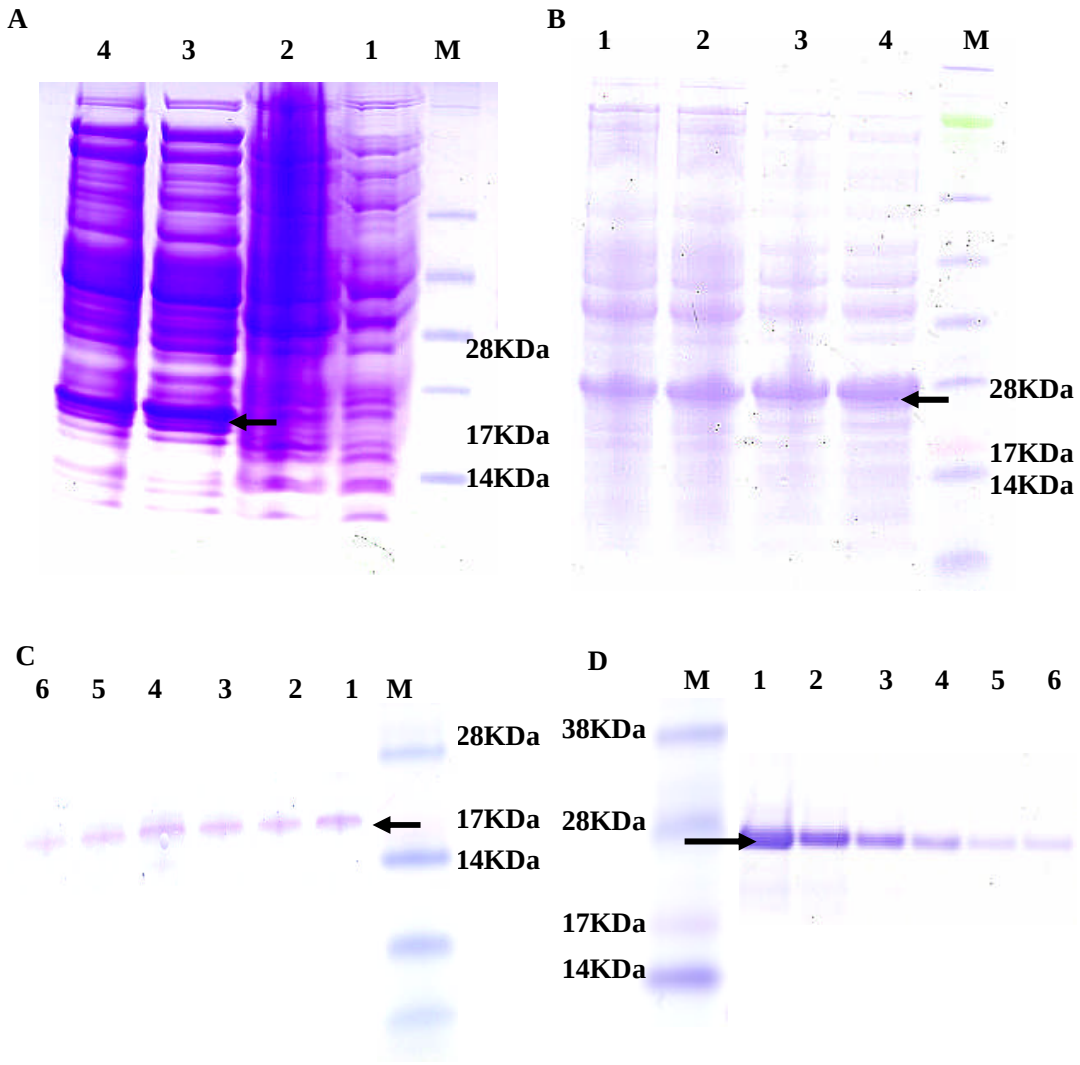


Fig. 4.6 SDS-PAGE electrophoresis of VAH and TPX at 200 V for 1 hour. (A). Protein expression of pET24a-VAH at 37℃ on different time points. M, See Blue plus 2 prestained standard (Invitrogen, UK). 1, uninduced empty pET24a. 2, pET24a-VAH 2 hour after 0.5 mM IPTG induction 3, pET24a-VAH 3 hour after 0.5 mM IPTG induction. 4, pET24a-VAH 4 hour after 0.5 mM IPTG induction. (B). Protein expression of pET29c-TPX at 37℃ on different time points. M, See Blue plus 2 prestained standard (Invitrogen, UK). 1, pET29c- TPX 1 hour after 1 mM IPTG induction. 2, pET29c-TPX 2 hour after 1 mM IPTG induction. 3, pET29c-TPX 3 hour after 1 mM IPTG induction. 4, pET29c-TPX 4 hour after 1 mM IPTG induction. (C) VAH protein eluted from His-binding column in the fractions. M, See Blue plus 2 prestained standard (Invitrogen, UK). 1-6: purified fractions. (D). TPX protein eluted from His-bounding column in the fractions. M, See Blue plus 2 prestained standard (Invitrogen, UK). 1-6: purified fractions. Molecular weight for VAH: 18.6 KDa; TPX: 26.4 KDa.

4.2.4 Identification of VAH and TPX by Western blotting

The VAH and TPX proteins were analyzed by Western blot using a mouse monoclonal anti-His antibody and sera from mice immunized with plasmids of pcDNA3.1-VAH and pcDNA3.1-TPX. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody. The proteins were visualized using Chemi Glow (1:1) reagents, and the membranes were exposed on a Fluor Chem^{TM}SP image machine. As shown in Fig 4.7, the VAH and TPX recombinant proteins were recognized by the sera from mice immunized with plasmids of pcDNA3.1-VAH or pcDNA3.1-TPX, respectively.

Fig. 4.7 Western blotting of VAH (A) and TPX (B) protein against sera from mice with immunized pcDNA3.1-VAH and pcDNA3.1-TPX vaccine, respectively. Protein was separated under reducing conditions on a 10-12% acrylamide gel. Gel was probed with sera from mice immunized with pcDNA3.1-VAH or pcDNA3.1-TPX vaccine with a dilution of 1:500.

4.2.5 Protein expression in COS7 cell and identification

1.25 μg DNA for each plasmid (pcDNA3.1-VAH, pcDNA3.1-TPX, pDEC-VAH, pISO-VAH, pDEC-TPX and pISO-TPX) was transferred into COS7 cells. Transfection lasted for 48 hours, then supernatants for secreted proteins and /or lysed cells for protein content were collected. Reverse transcription PCR and Western blotting were carried out to identify whether the plasmid expressed in COS7 cell or not. Results showed that transcripts of all plasmids were detected using VAH- or TPX-specific primers (Fig. 4.8) and all proteins expressed in COS7 cells could be recognized strongly by the serum from the VAH- or TPX DNA immunized mice (Fig. 4.9).

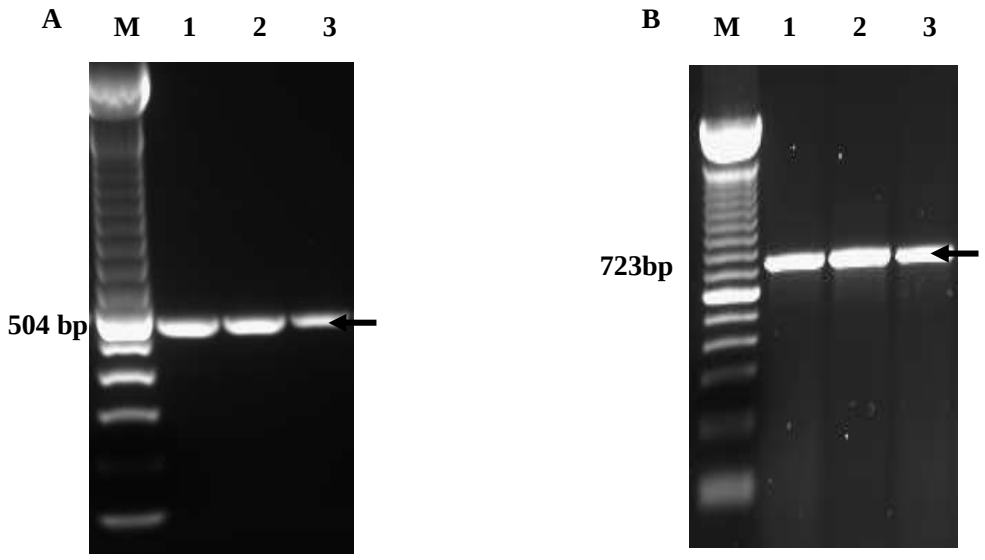


Fig. 4.8 Agarose gel electrophoresis of RT-PCR identification of gene expression in COS7 cells in 0.5% TBE at 120 V for 40 minutes. Plasmids of VAH, and TPX were transfected into COS7 cells in vitro, then cells were harvested and ultrasonicated, mRNA were extracted to carry out the RT-PCR. (A). M: 100bp DNA ladder; 1, pcDNA-VAH; 2: pDEC-VAH; 3: pISO-VAH; (B) M: 100bp DNA ladder; 1, pcDNA-TPX; 2, pDEC-TPX; 3, pISO-TPX.

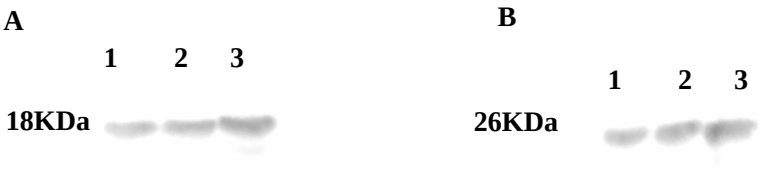
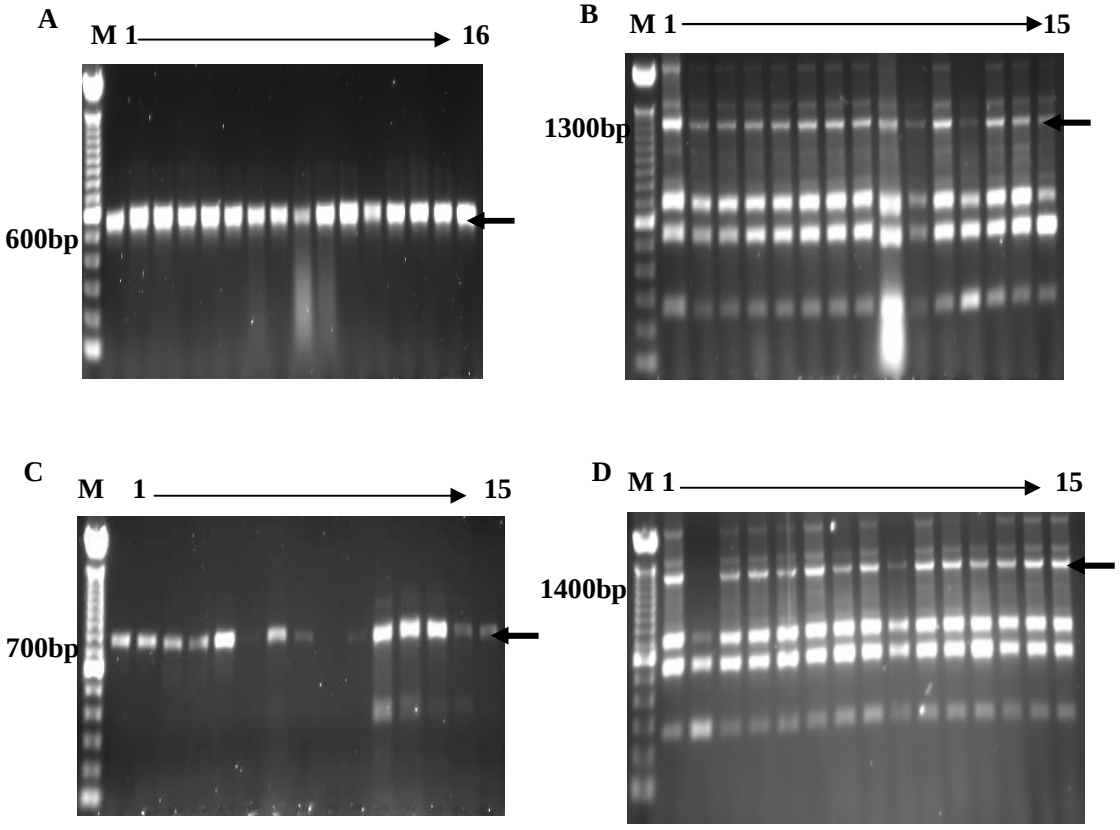


Fig. 4.9 Western blotting identification of proteins expression in COS7 cells under reducing conditions on a 10-12% acrylamide gel at 30 V for 1 hour. Plasmids of VAH and TPX were transfected into COS7 cells *in vitro*, then cells were harvested and ultrasonicated, the supernatants were used to perform the Western blotting against the serum from the VAH TPX DNA vaccinated mice. (A).Western blotting of VAH; 1, pcDNAVAH; 2, pDECVAH; 3, pISOVAH; (B).Western blotting of TPX; 1, pcDNATPX; 2, pDECTPX; 3, pISOTPX.

4.2.6 Tissue distribution of VAH / TPX plasmids in mice

To determine the distribution of plasmids of VAH and TPX in mice following i.m injection and electroporation, two doses of plasmid were given at 2-week interval and necropsy was

performed at 28 days after the final immunization. Mouse tissues were collected from muscle at the point of inoculation, spleen, liver and lung and prepared for mRNA extraction. Reverse transcription PCR with VAH- and TPX-specific primers and T7/ BGH primers (which locate the flank of inserted gene on the plasmids, implying the existence of plasmids in tissues). The results showed that when using the specific antigen primers all genes were expressed in muscle, spleen, liver and lung. In addition, all genes were detected in muscle, spleen, liver and lung when the T7/ BGH plasmid specific primers were used (Table 4.1). VAH appeared to be expressed more intensively in lung, spleen and liver than in in muscle while TPX was expressed equally by muscle, spleen, lung and liver. The presence of the carrier plasmids was detected more easily in spleen, lung and liver rather than in muscle. Fig 4.10 represents four examples for the tissue distribution *in vivo*. The overall tissue distributions of VAH/ TPX vaccines are listed in (Table 4.1).



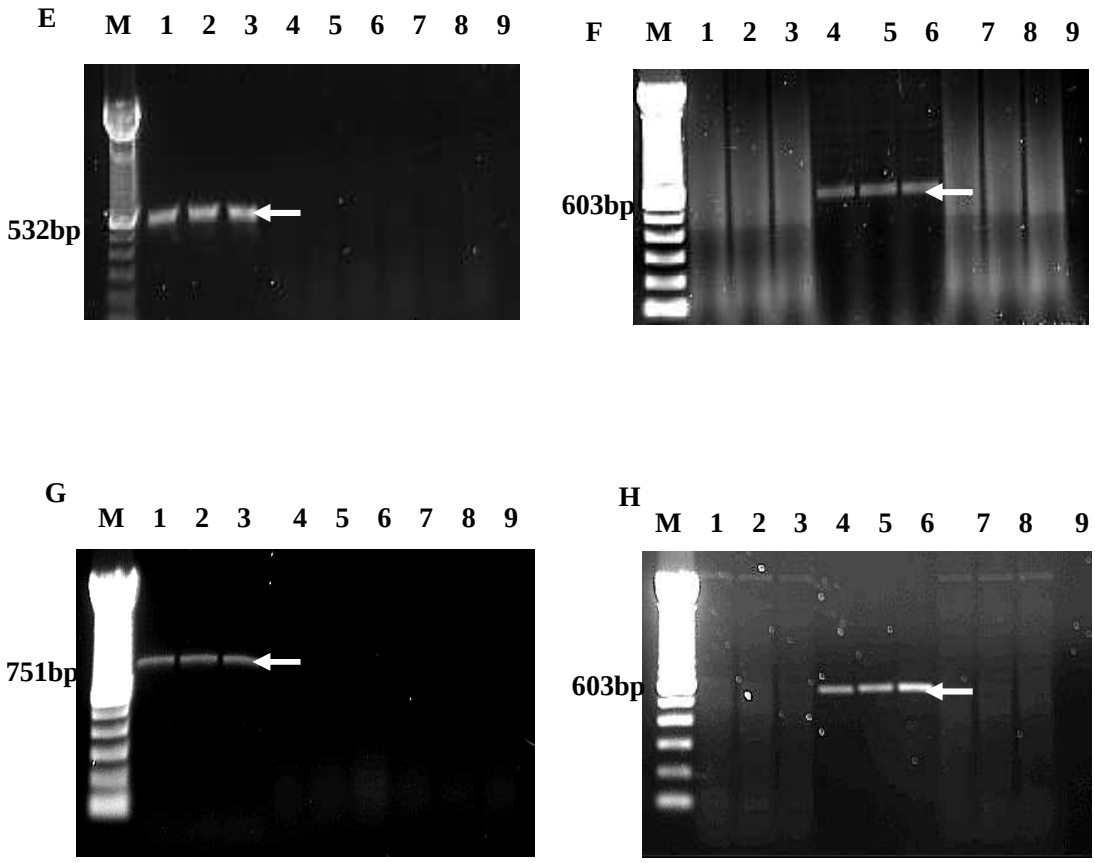


Fig.4.10 Agarose gel electrophoresis of examples of tissue distribution of VAH/TPX plasmids in mice in COS7 cells in 0.5% TBE at 120 V for 40 minutes. All plasmids of VAH and TPX were injected into muscle twice followed by the electroporation; 28 days later, muscle, spleen, liver and lung were assayed to detect the gene distribution by specific primers and T7/BGH primers by reverse transcriptional PCR. (A). pcDNAVAH distribution in spleen and muscle detected by VAH-specific primers. (B). pDECVAH distribution in lung detected by T7/BGH primers. (C). pcDNATPX distribution in muscle detected by TPX-specific primers. (D). pDECTPX distribution in muscle and spleen detected by T7/BGH primers. (E) VAH distribution in muscle detected by VAH-specific primers; 1-3: VAH; 4-6: OVA ; 7-9: Naïve; (F) OVA distribution in muscle detected by OVA-specific primers; 1-3: VAH; 4-6: OVA ; 7-9: Naïve; (G) TPX distribution in muscle detected by TPX-specific primers; 1-3: TPX; 4-6: OVA ; 7-9: Naïve;(H) OVA distribution in muscle detected by OVA-specific primers; 1-3: TPX; 4-6: OVA ; 7-9: Naïve;

Table. 4.1 Summary of tissue distribution of VAH and TPX vaccines in mice detected by RT-PCR (for detection of expressed sequence) and standard PCR (for detection of existence of plasmids)

plasmid	Specific primers				T7/BGH primers			
	Muscle	spleen	lung	liver	Muscle	spleen	lung	liver
pcDNAVAH2	+	++	+++	++	+	+	+	+
pDECVAH2	+	++	+++	++	+	+	+	+
pISOVAH2	+	++	+++	++	+	+	+	+
pcDNATPX4	+	+	+	+	+	+++	++	++
pDECTPX4	+	+	+	+	+	+++	++	++
pISOTPX4	+	+	+	+	+	+++	++	++
Naïve	-	-	-	-	-	-	-	-

"+"represents Expression positive; "-"represents no expression

4.2.7 Higher levels of Th1 and Th2 antibodies were evoked by DEC-VAH compared to control

In order to compare the capability of DEC-VAH and pcDNA-VAH to provoke and enhance immune responses, five experimental mouse groups were tested. These comprised: naïve, primary infection, pempty, pcDNA-VAH and pcDNA-DEC-VAH. Naïve, primary infection and pempty groups were the negative controls to pcDNA-VAH and DEC-VAH. On D0 and D14, mice were given two doses of vaccine in addition of the plasmids encoding IL4, Flt3L and MIP1α as adjuvants. The electroporation procedure was performed following each injection. Mice were challenged with 40 L3s 35 days post the final immunization followed by necropsy 60 days post challenge. Blood (for sera preparation and Mf counting), pleural cavity lavage (for cell counting, cytospin, and cytokine detection) and lymph node (for cell stimulation *in vitro* and cytokine detection) were collected.

ELISA analysis showed that high levels of IgG1 with a titer of over 100,000 (in Log10) were induced by pcDNA-VAH and DEC-VAH. Significantly stronger IgG1 responses were produced by both pcDNA-VAH and DEC-VAH in contrast to primary infection ($p=0.04$, 0.01, respectively, see Fig. 4.11 A). However, although there was no significant difference

between pcDNA-VAH and DEC-VAH groups, DEC-VAH induced more IgG1 than pcDNA-VAH.

Levels of IgG2a were nearly equal to those of IgG1 (100,000) following vaccination with both pcDNA-VAH and DEC-VAH. More importantly, pcDNA-VAH and DEC-VAH induced significant higher IgG2a in contrast to primary infection (Fig. 4.11 B).

DEC-VAH induced higher levels of IgE compared to primary infection and pcDNA-VAH groups but pcDNA-VAH failed to induce greater levels of IgE than controls (Fig. 4.11C).

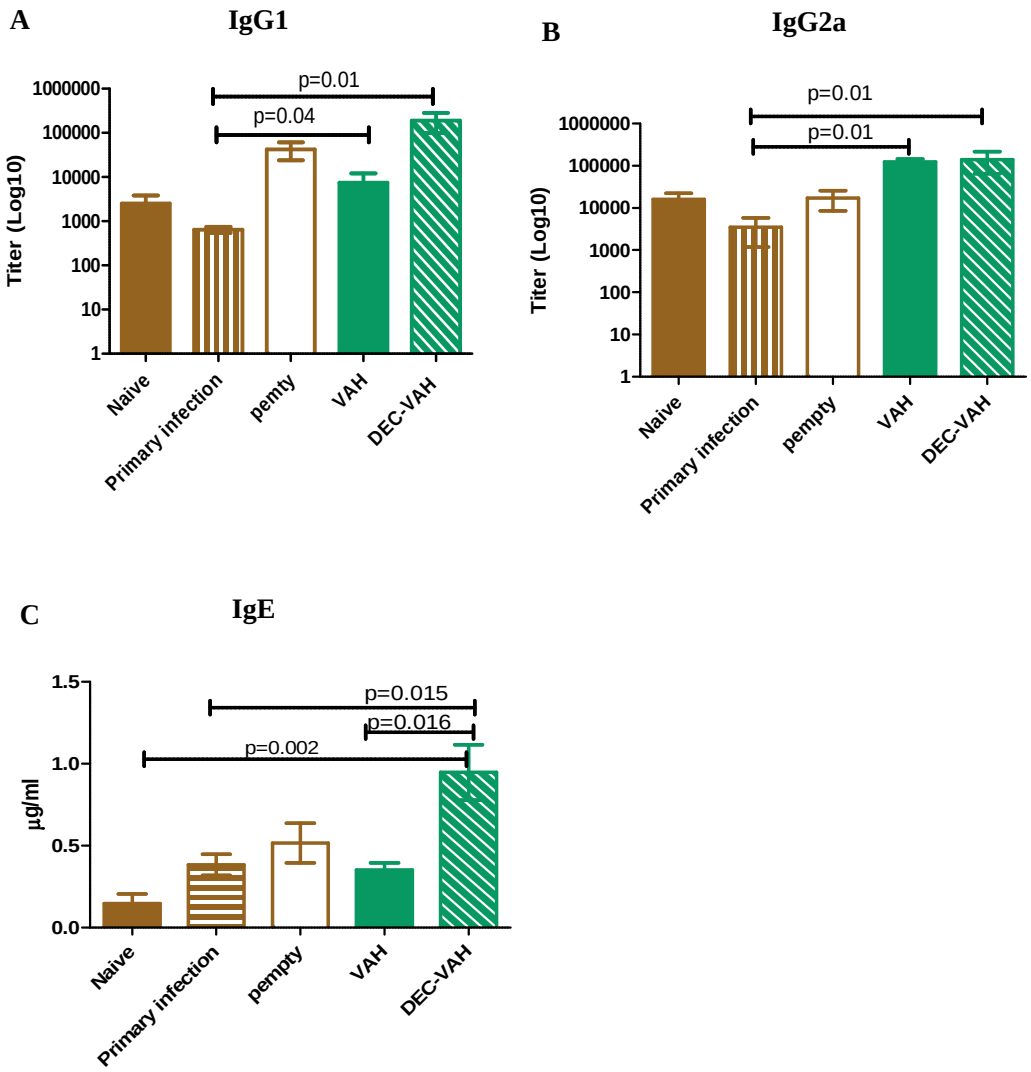


Fig. 4.11 DEC-VAH evoked higher levels of antibodies than VAH. Antibody levels of IgG and IgE were compared between pcDNA3.1-VAH and DEC-VAH by indirect ELISA. All test groups were co-administrated the plasmids encoding IL4, Flt3L and MIP1α as adjuvants. Results are shown as titer of IgG and the mean of replicate samples (+/- S.E.M) for total IgE. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise. 5 mice per group were tested.

4.2.8 DEC-VAH induced significant quantities of IL 4, IL5 and IL13 in pleural cavity fluid

To investigate the cytokine profile produced by DEC-VAH vaccination, a capture ELISA was used to detect the expression of cytokines in pleural cavity fluid 60 days post challenge.

Various cytokines including IL4, IL5, and IL13 were measured. Significantly higher IL4 was induced by DEC-VAH vaccination in contrast to the primary infection ($p=0.013$) and pempty ($p=0.03$) groups. Although the level of IL4 induced by pcDNA-VAH decreased compared to DEC-VAH, it was still stronger than the controls (Fig. 4.11 A). For IL5, the same trend as IL4 was seen and a significant difference ($p=0.04$) between DEC-VAH and primary infection but not pempty group was demonstrated (Fig. 4.11 B). The IL13 expression profile was also similar to IL4 and IL5, with significantly higher production by DEC-VAH than primary infection and pempty ($p=0.02$ and $p=0.01$, respectively. Fig. 4.11 C).

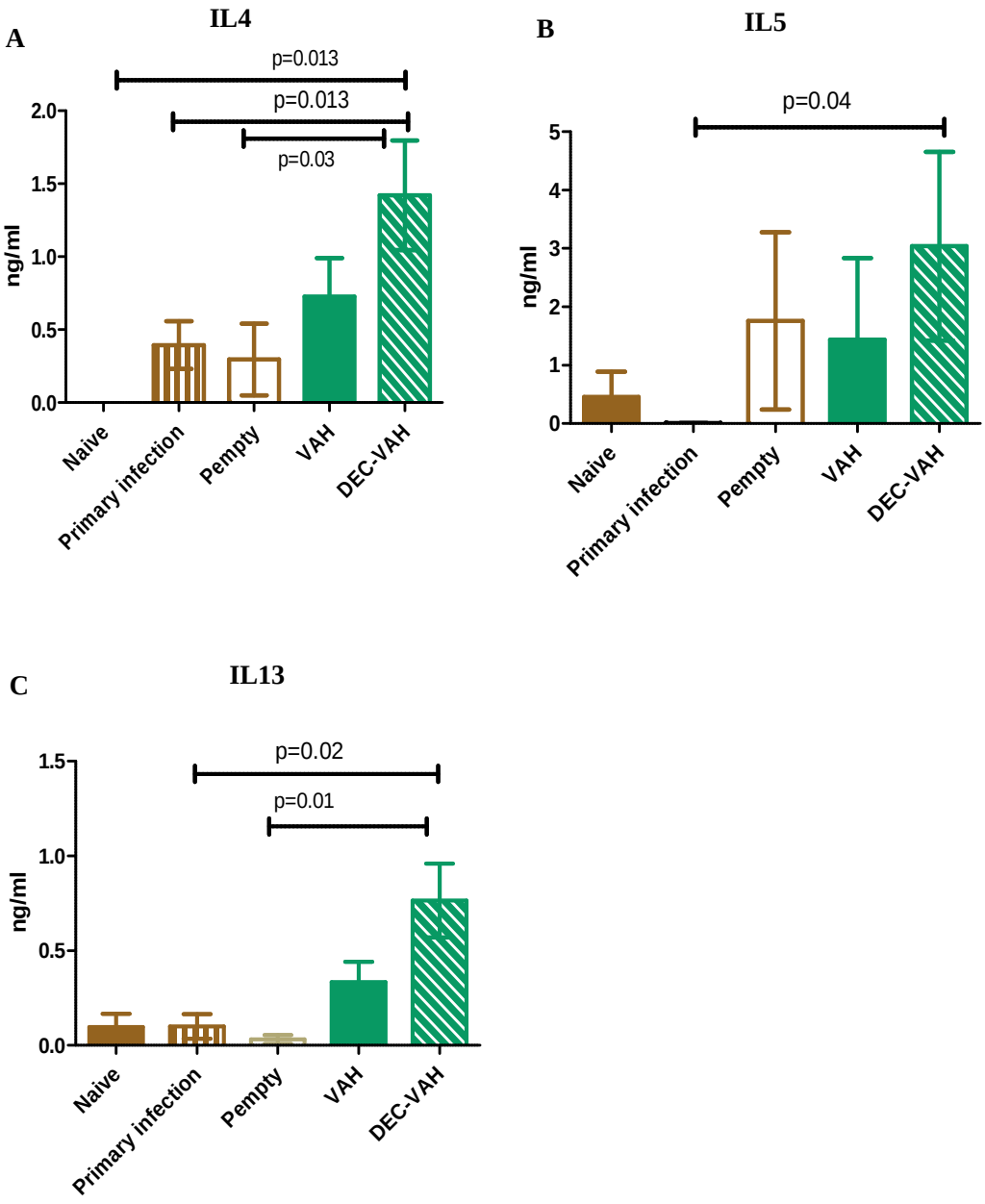


Fig.4.11. DEC-VAH induced significant quantities of IL 4, IL5 and IL13 in the pleural cavity fluid. Supernatants of pleural cavity lavage were harvested and the level of various cytokines (IL4, IL5, IL13) were measured by sandwich ELISA-(see methods and materials). Results are shown as the mean of replicate samples. Kruskal –Wallis and Mann-Whitney tests were used as statistical methods. Differences were considered significant for $p < 0.05$; p values were not reported otherwise. 5 mice were tested in each group.

4.2.9 Vaccination with DEC-VAH resulted in decreased levels of IFNγ in the pleural cavity and decreased IL10 production by lymph nodes

The Th1 cytokine IFNγ and Treg/ Th2 cytokine IL10 were also measured to assess the cellular immune effect of pDEC-VAH vaccination. The production of IFNγ displayed a decreased trend. Both pcDNA-VAH and DEC-VAH induced quite low levels of IFNγ with the lowest after DEC-VAH vaccination. However, primary infection produced the most IFNγ (Fig.4.12 A). Both pcDNA-VAH and DEC-VAH failed to produce IL10 in the pleural cavity lavage. However, in lymph node culture, significantly lower IL10 was induced by DEC-VAH compared to primary infection (p=0.009) and pempty (p=0.018) groups (Fig.4.12 .B).

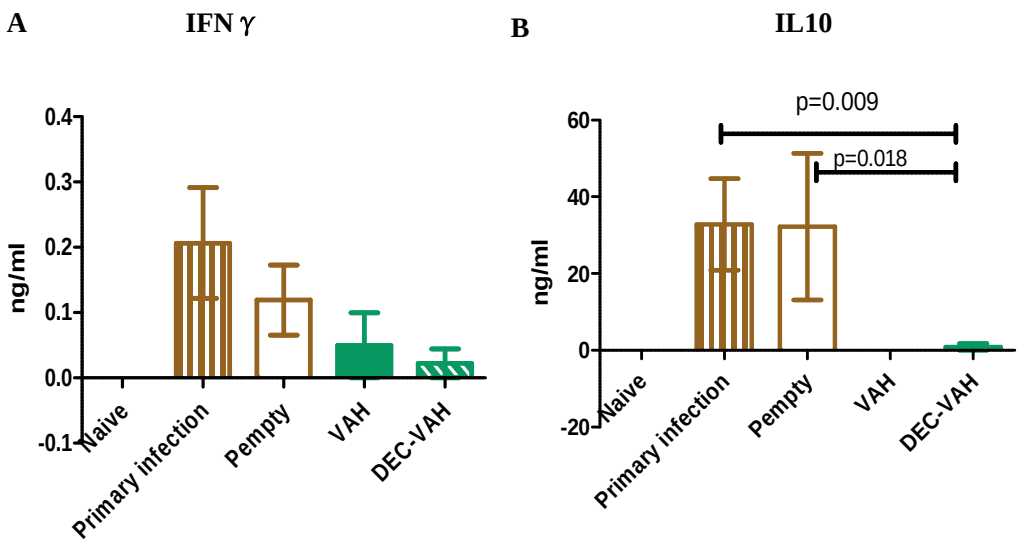


Fig.4.12. Vaccination with DEC-VAH resulted in reduced IFNγ in the pleural cavity and decreased IL10 in lymph node culture. Supernatants of pleural cavity lavage and lymph node culture were harvested and the levels of IFNγ and IL10 were measured by capture ELISA-(see methods and materials). The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise. Results are shown as the mean of replicate samples (+/- S.E.M). 5 mice were tested in each group.

4.2.10 Increased Th2 cytokines and IFNγ were induced with stimulation of Ls-Ag *in vitro* by DEC-VAH immunization

Cytokine profiles in supernatants of lymph node cell culture following stimulation with Ls-Ag were measured. Cytokines in medium were measured as background controls. Results showed that in medium, IL4 production after vaccination with VAH was higher than after immunization with DEC-VAH and controls. In contrast, the level of IL5 induced by vaccination with VAH decreased compared to immunization with DEC-VAH. However, IL13 and IFNγ were poorly produced by the vaccinations. In contrast, cytokines in supernatants of lymph node culture following stimulation with Ls-Ag showed that IL4 production in response to Ls-Ag decreased in the VAH group compared to in mice immunized against DEC-VAH, but the quantity decreased when compared to that in medium. The level of IL5 showed similar trends to that in medium but quantities in both groups of VAH and DEC-VAH were reduced. Levels of IL13 in both groups of VAH and DEC-VAH increased compared that in medium. There was no increase in production of IFNγ in both groups of VAH and DEC-VAH compared to that in medium, although the levels of IFNγ increased a little in both naïve and primary infection groups (Fig.4.13).

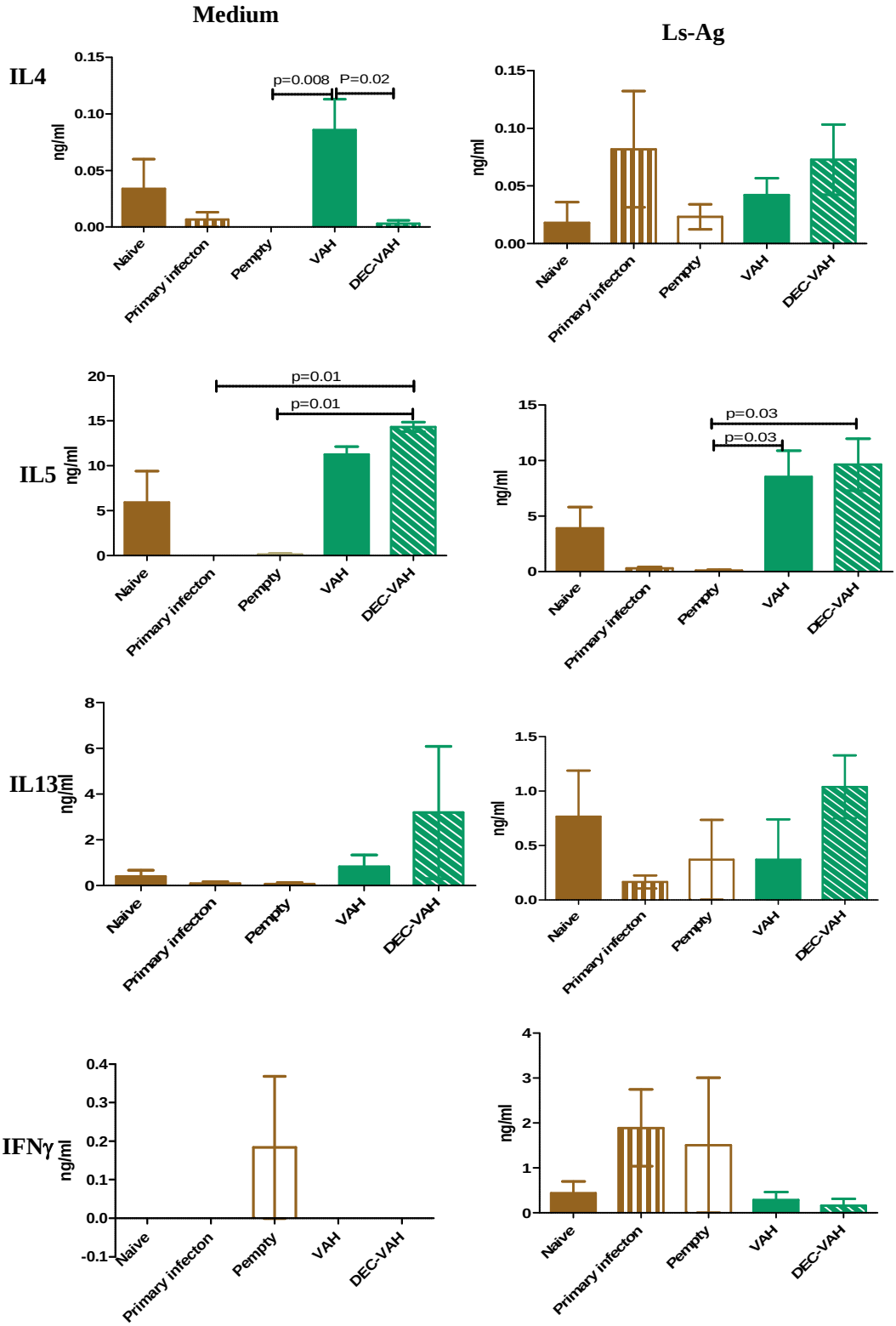


Fig. 4.13 Increased Th2 cytokines and IFNγ were induced by DEC-VAH vaccination with stimulation of Ls-Ag in vitro. Supernatants of lymph node cell cultures were harvested 24 hours post stimulation with *L. sigmodontis* secretary antigen. The level of IL4, IL5, IL13 and IFNγ were measured by sandwich ELISA- (see methods and materials). Results are shown as the mean of replicate samples (+/- S.E.M). The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise. 5 mice per group were tested.

4.2.11 Elevated numbers of eosinophils, but not macrophages and neutrophils, were detected following vaccination with DEC-VAH

Numbers and types of cells from pleural cavity were recorded following concentration using a cytospin assay. The numbers of all cells recovered from the pleural cavity following a primary infection, or vaccination with the carrier plasmid, or recombinant vaccines, were similar but all were elevated compared to naïve controls. Eosinophil numbers in all experimental groups were higher than measured in the naïve control group. Mice immunized with pcDNA-VAH and DEC-VAH had the highest number of eosinophils but these levels were not significantly different from those recorded for the primary infection and control group immunized with the carrier plasmid. A similar situation was seen with respect to neutrophil numbers.

In the case of macrophages, again, the numbers in all experimental groups were higher than naïve controls but in this case those mice immunized with pcDNA-VAH or DEC-VAH had lower numbers than seen in the primary infection or the group immunized with the carrier plasmid, although no significant differences were seen (Fig. 4.14).

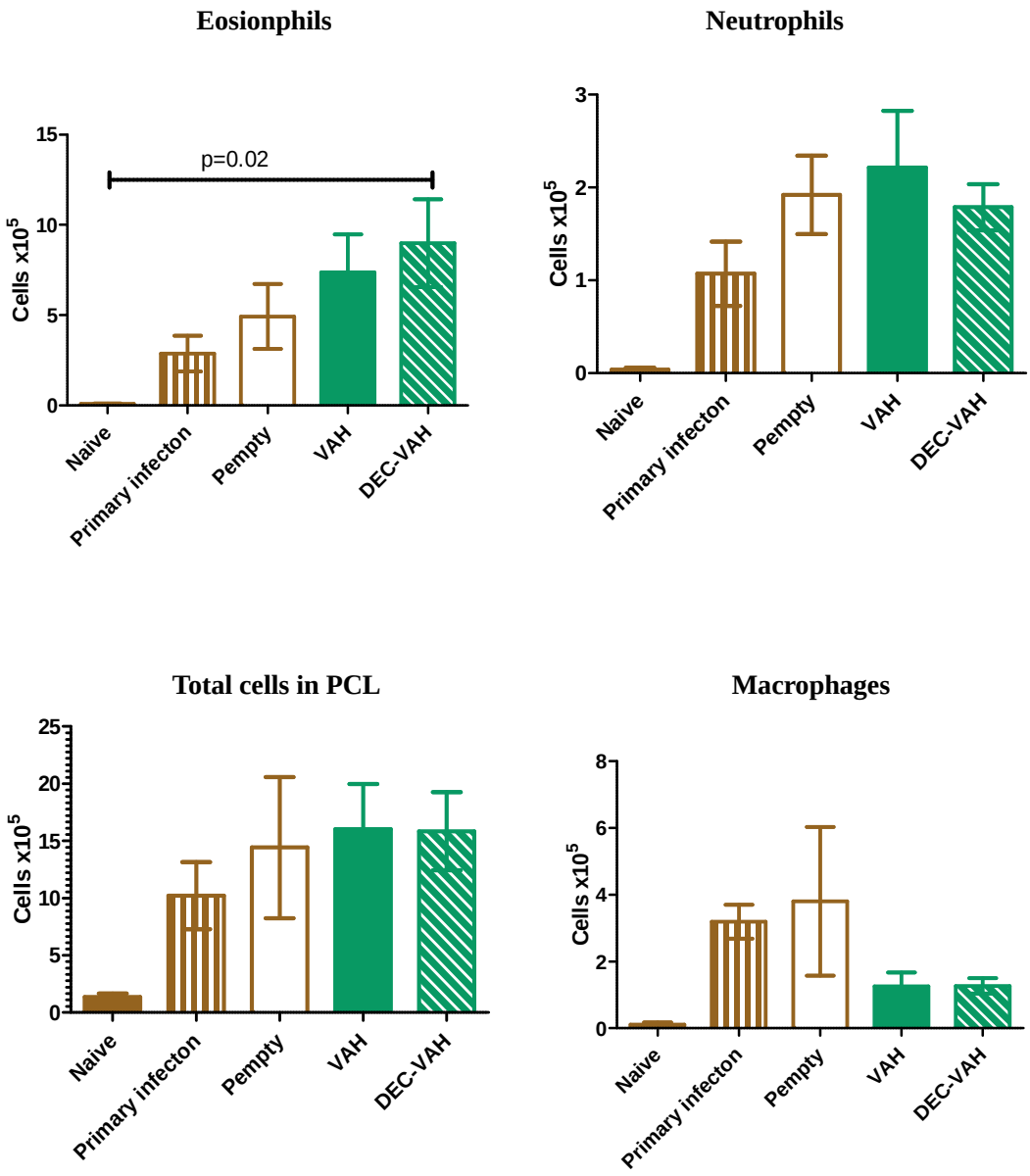


Fig. 4.14 Elevated numbers of eosinophils, but not macrophages nor neutrophils, were induced by DEC-VAH vaccination. Total cell numbers were determined using the CASY model TT cell counter system. Eosinophil, neutrophil and macrophage numbers were determined following concentration by cytospin and enumerated by microscopy on fixed slides (300 cells, at minimum on each slide were counted). 5 mice were tested in each group. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

4.2.12 Vaccination with DEC-VAH resulted in reduced worm burdens

To assess the protection elicited by the DEC-VAH vaccination, worms in the pleural cavity and Mf in blood were counted. Each mouse had been challenged with 40 L3s. Protection was recorded as a reduction in worm numbers in test animals compared to those in the control groups. As presented in Fig.4.15. A, the number of worms recovered from DEC-VAH immunization decreased when compared to that of primary infection group, although similar number of worms was recovered from pcDNA-VAH group. As far as the number of Mf recovered in the blood was concerned, a low level of recovery was displayed in all tested groups except the pempty group in which only one mouse carried a high microfilaraemia with over 450 in 30 µl blood.

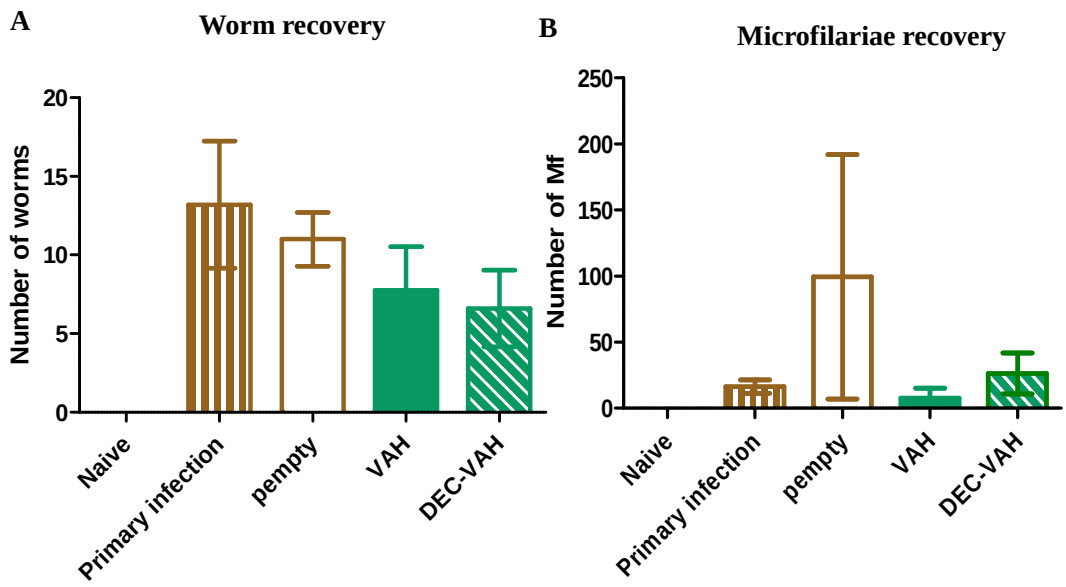


Fig. 4.15. Vaccination with DEC-VAH resulted in reduced worm burden. Necropsy was performed 60 day post challenge, pleural cavity lavage was collected and worms were fixed with 70% hot ethanol; 30 µl blood were added into 270 µl FACS lysing solution then microfilariae were counted under microscope as well as adult worms. Results are shown as the mean of replicate samples (+/- S.E.M). 5 mice were tested in each group. Mann-Whitney test was used as a statistical method. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

4.2.13 Th2-dominant antibodies were produced by DEC-TPX vaccination

To compare the ability of DEC-TPX and pcDNA-TPX to evoke and enhance the immune responses, six groups of mice were investigated: naïve, primary infection, pempty, pISO-TPX, pcDNA-TPX and DEC-TPX. Naïve, primary infection pempty and pISO-TPX were used as negative controls to pcDNA-TPX and DEC-TPX. Mice were immunized twice on D0 and D14 with DNA vaccine candidates together with plasmids encoding IL4, Flt3L and MIP1α as adjuvants. Electroporation was performed at the site of inoculation following each injection. Mice were challenged with 25 L3s on D28 after the final immunization and the necropsy was carried out 60 days post challenge. Blood (for sera preparation and Mf counting), pleural cavity lavage (for cell counting, cytospin and cytokine detection) and lymph node (for cell stimulation *in vitro* and cytokine detection) were collected.

The levels of IgG1 induced by pcDNA-TPX and DEC-TPX were higher than naïve control. However, the DEC-TPX showed a stronger ability to enhance IgG1 production compared to pcDNA-TPX which induced only half the level of IgG1 of DEC-TPX (Fig.4.16 A).

As far as IgG2a was concerned, it was induced by DEC-TPX but not by pcDNA-TPX. However, IgG2a levels induced by DEC-TPX were lower than those induced by pISO-TPX and pempty (Fig.4.16 B).

For total IgE level, there was an upward trend among groups as shown in Fig.4.16 C. Both pcDNA-TPX and DEC-TPX induced greater levels of total IgE than controls, and DEC-VAH group induced significant increase of total IgE in contrast to the primary infection group ($p=0.03$, Fig. 4.16 C).

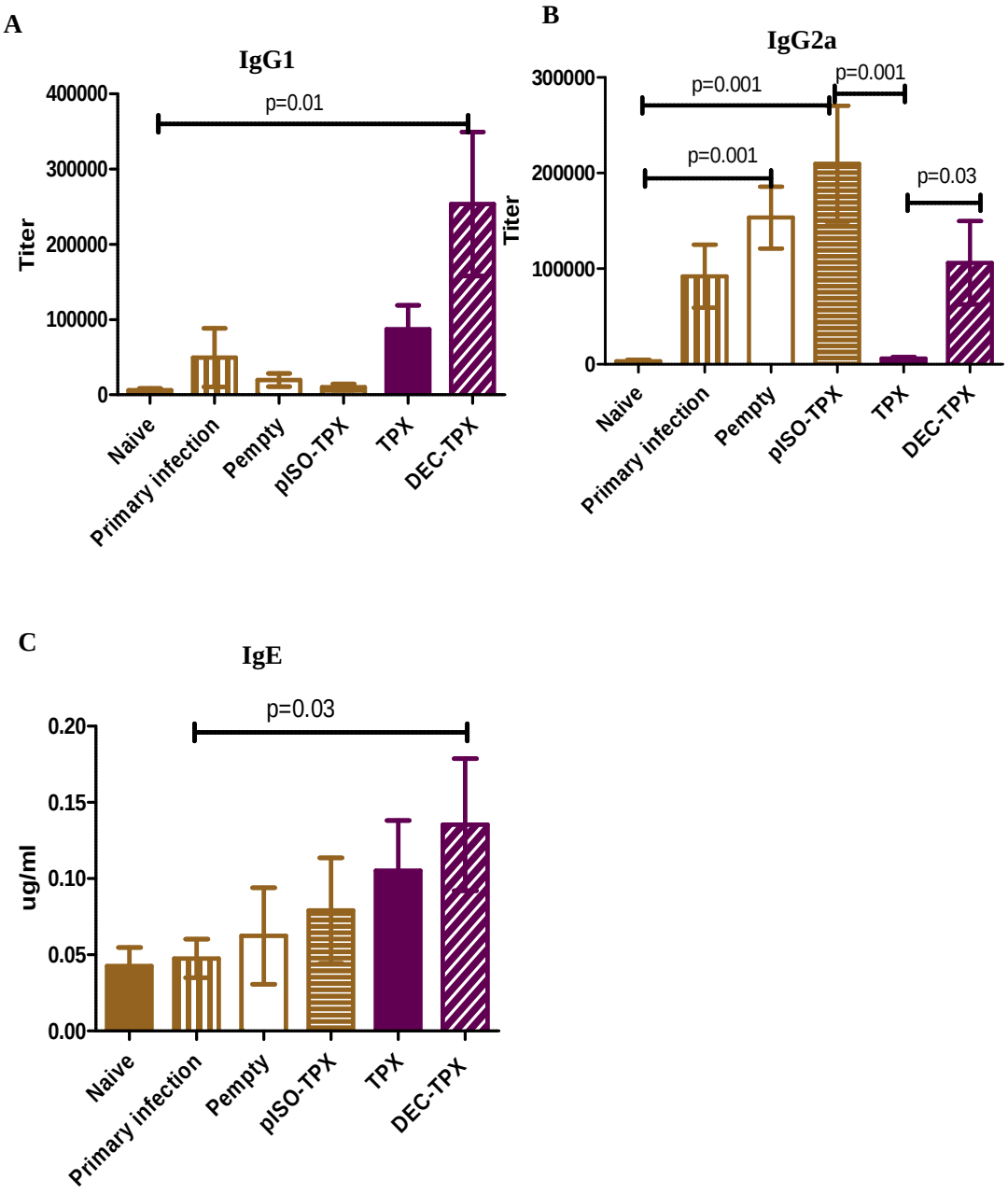


Fig.4.16. Th2-dominant antibodies were produced by DEC-TPX vaccination. Antibody levels of IgG and IgE were compared among pDEC-TPX and the pcDNA-TPX by indirect ELISA coated with recombinant TPX protein (see methods and materials). All test groups were immunized with the adjuvants IL4, Flt3L and MIP1α.Results are shown as titer of IgG and the mean of replicate samples (+/- S.E.M) for total IgE. Kruskal-Wallis and Mann-Whitney tests were used as statistical methods. 5 mice were tested in each group. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

4.2.14 IL5 and IL13 rather than IL4 in the pleural cavity fluid were enhanced by DEC-TPX immunization

To assess the impact of DEC-TPX vaccination on the production of cytokines, the cytokines in the pleural cavity were detected by capture ELISA. In the pleural cavity, an up-regulation of IL5 and IL13 was demonstrated in mice immunized with DEC-TPX in contrast to pcDNA-TPX. IL4 was undetectable when immunized with DEC-TPX (only one mouse produced IL4) or pcDNA-TPX (Fig. 4.17 A, B, C).

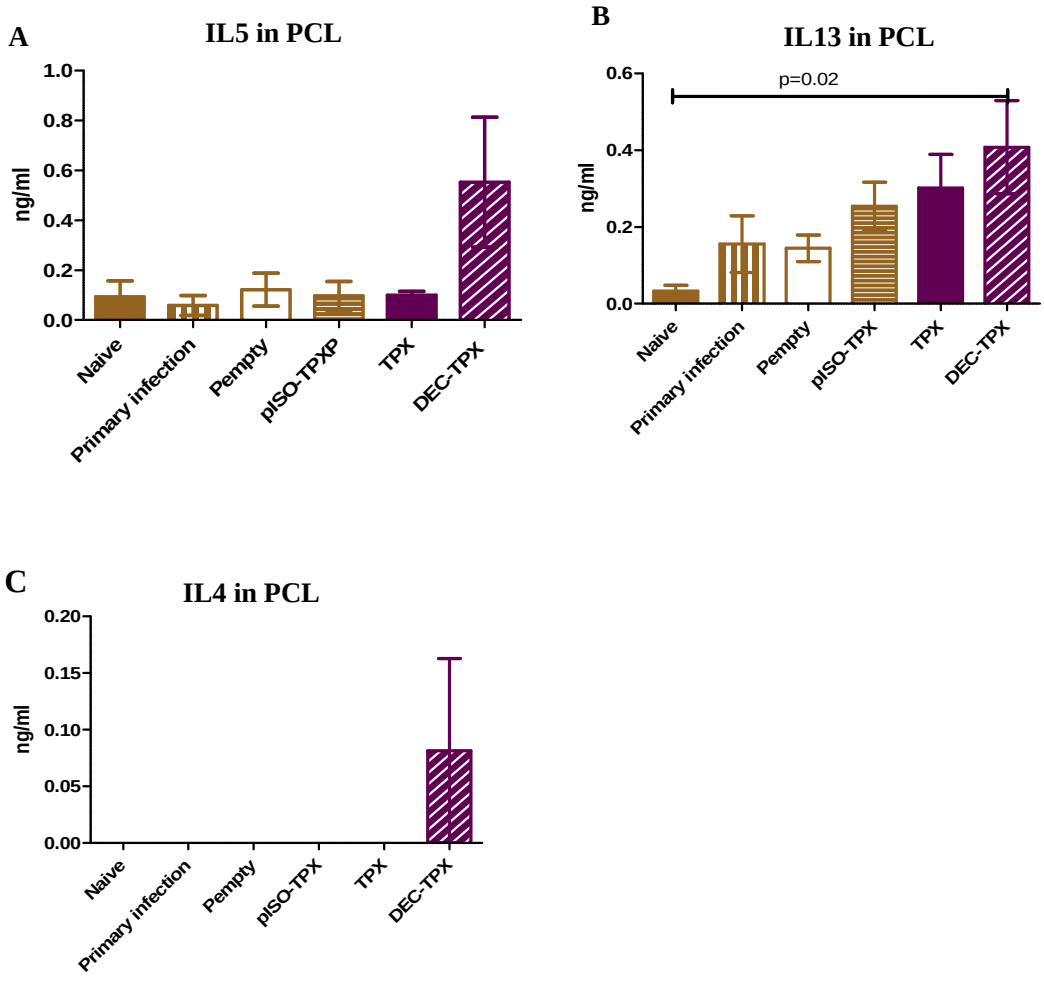


Fig. 4.17 IL5 and IL13 rather than IL4 were enhanced by DEC-TPX immunization. Supernatants of the pleural cavity lavage were harvested and the levels of various cytokines (IL5, IL13 and IL4) we re measured by capture ELISA- (see methods and materials). Results are shown as the mean of replicate samples (+/- S.E.M). 5 mice were tested in each group. . The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

4.2.15 Increased IFNγ in the pleural cavity fluid was induced by TPX DNA vaccination

The level of IFNγ was measured by the capture ELISA to evaluate the Th1 cellular immune effect of TPX DNA vaccination. In the pleural cavity lavage, increased levels of IFNγ were induced by DEC-TPX compared to the pcDNA-TPX group but all were elevated compared with control groups including the primary infection. The expression of IFNγ was sharply increased on DEC-TPX with comparison to pempty (p=0.08) and the primary infection group (p=0.016) (Fig. 4.18).

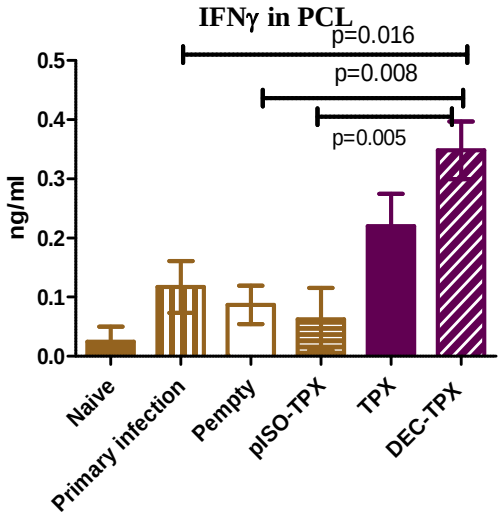


Fig. 4.18 Increased IFNγ in the pleural cavity fluid was induced by TPX DNA vaccination. Supernatants of pleural cavity lavage were harvested and the level of IFNγ was measured by sandwich ELISA- (see methods and materials). Results are shown as the mean of replicate samples (+/- S.E.M). The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise. 5 mice per group were tested.

4.2.16 Levels of IL10 were no difference between primary infections and mice vaccinated with DEC-TPX

The level of IL10 was measured by capture ELISA to evaluate the Th2/Treg cellular immune effect of TPX DNA vaccination. In pleural cavity lavage, the levels of IL10 detected in mice

immunized with pcDNA-TPX and DEC-TPX were no significant difference (Fig.4.19).

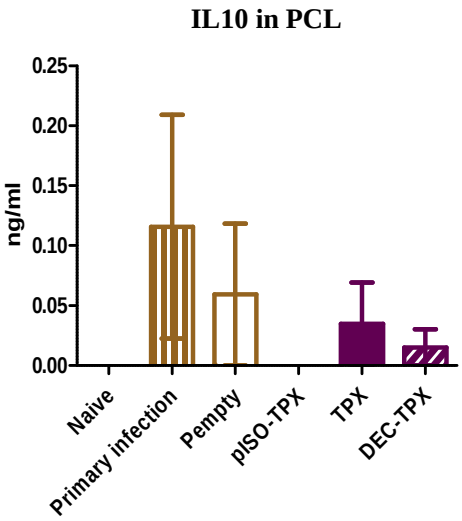
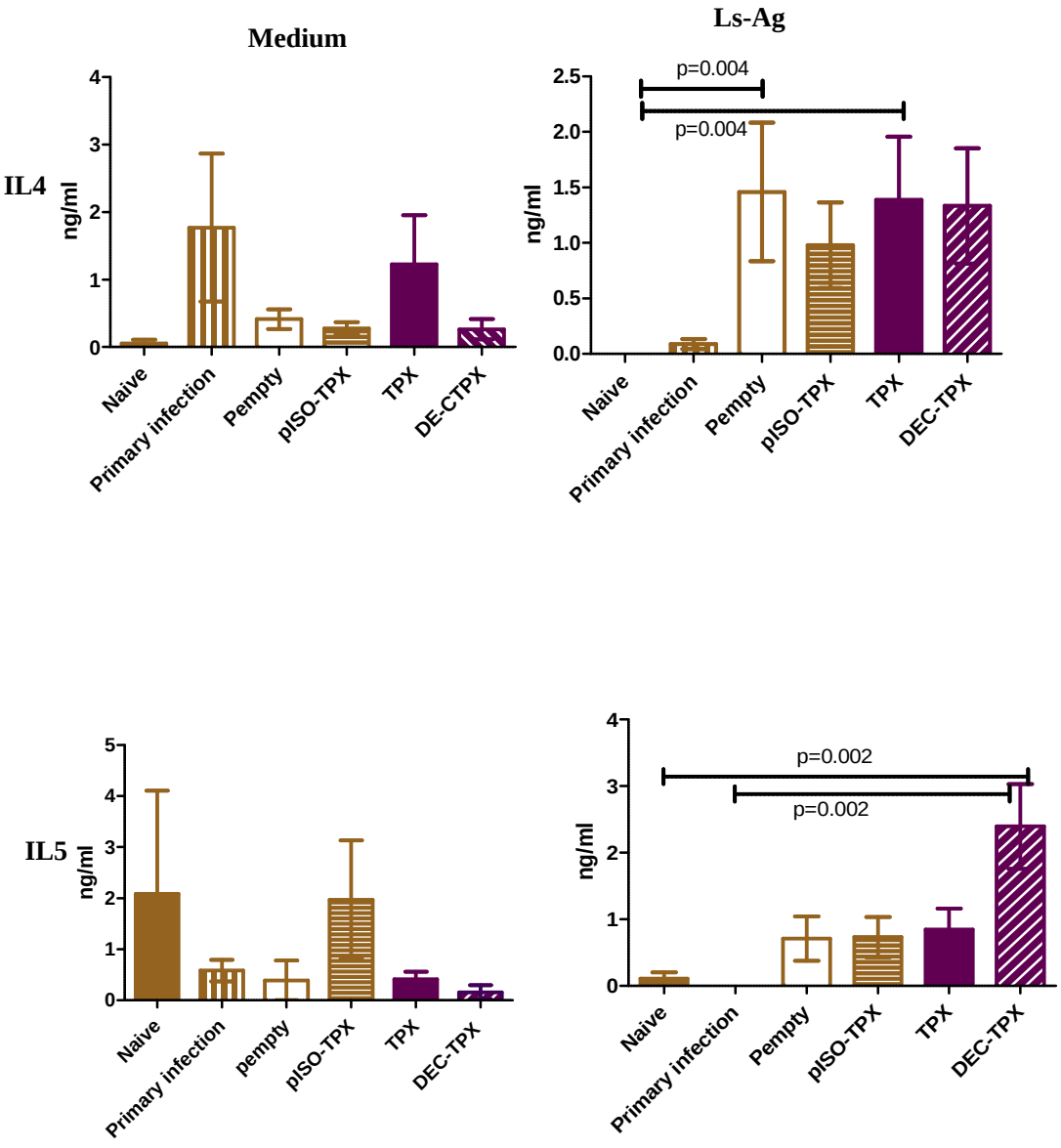


Fig. 4.19 Levels of IL10 were no significant difference between primary infections and mice vaccinated with DEC-TPX. Supernatants of pleural cavity lavage were harvested and the level of IL10 was measured by sandwich ELISA- (see methods and materials). Results are shown as the mean of replicate samples (+/- S.E.M). 5 mice were tested in each group. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

4.2.17. Lymph nodes cells produced more IL5, IL13 and IFNγ than IL4 and IL10 following stimulation with Ls-Ag *in vitro*

Cytokine profiles of supernatant fluids collected from lymph node culture following stimulation of Ls-Ag were measured. IL4 production was increased over control levels in all test groups although there was no difference in the amount of cytokine induced by DEC-TPX, TPX and pempty vaccinations. The induction of antigen-specific IL4 by the pempty group was unexpected and this apparent abnormality has not been explained. When IL5 and IL13 levels were measured, again enhanced responses were seen in the experimental groups but in this case, levels measured in mice immunized with DEC-TPX showed a considerable increase over primary infection and naïve groups. However, statistical analysis revealed that the only significant difference in cytokine levels was that of IL13 between DEC-TPX and the primary infection ($p=0.009$).

The induction of antigen-specific IL10 levels by the DEC-TPX and TPX were lower than that induced by pISO-TPX. Enhanced IFNγ responses were seen in experimental groups of DEC-TPX compared to TPX, primary infection and pempty groups. However, stimulation of IFNγ by pISO-TPX vaccination was unexpected and this apparent abnormality has not been explained.



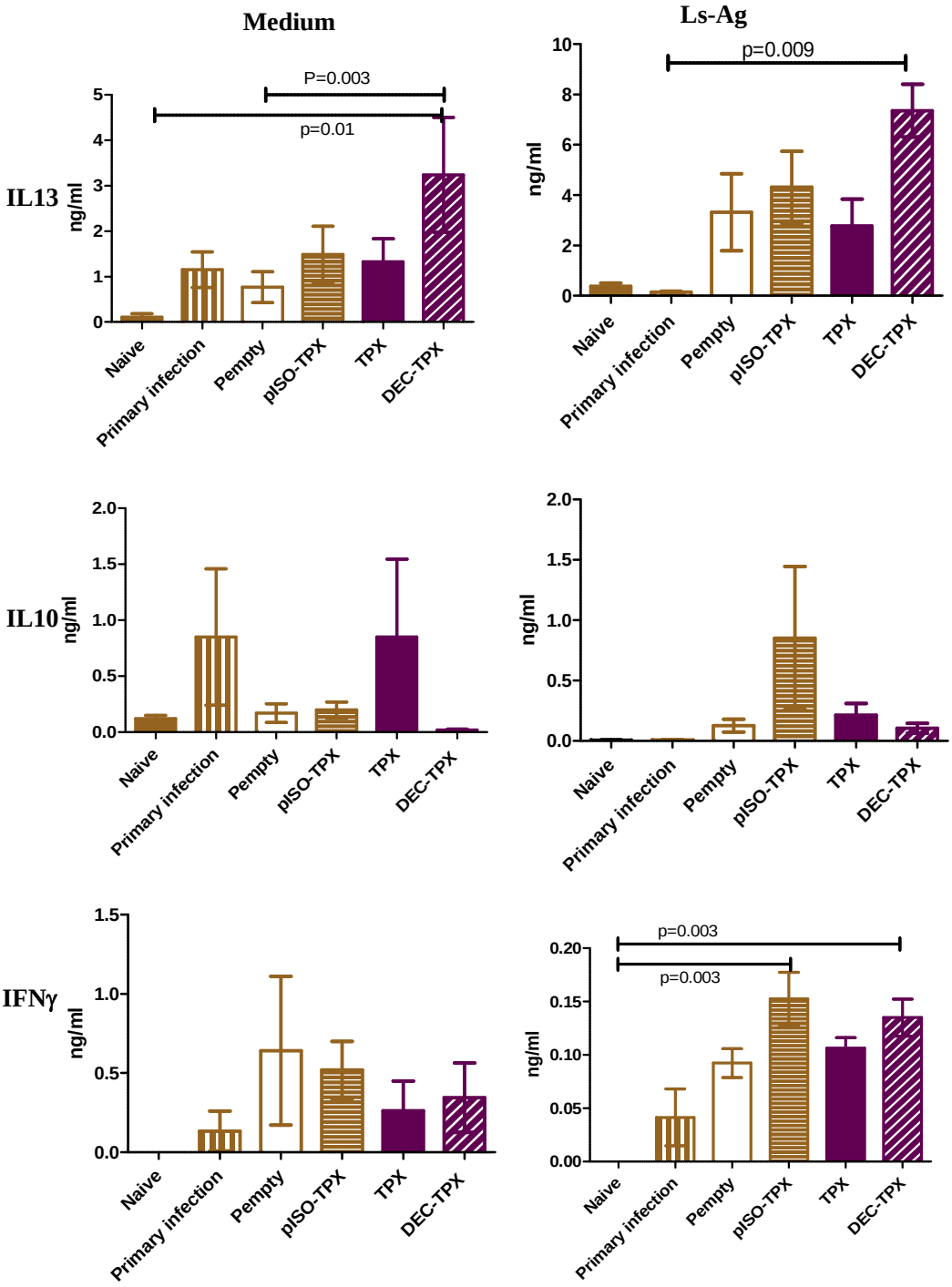


Fig. 4.20 IL5, IL13 and IFNγ rather than IL4 and IL10 were unregulated following stimulation of Ls-Ag in vitro by DEC-TPX vaccination. Supernatants of lymph nodes culture were harvested 24 hours post stimulation of *L.sigmodontis* secretory antigen. The level of IL4, IL5, IL10, IL13 and IFNγ were measured by sandwich ELISA- (see methods and materials). Results are shown as the mean of replicate samples (+/- S.E.M). 5 mice were tested in each group. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

4.2.18 Lower numbers of eosinophils, macrophages and neutrophils were recruited in the pleural cavity with DEC-TPX immunization

The types and numbers of cells recovered from the pleural cavity of experimental mice were determined. Vaccination with pcDNA-TPX and DEC-TPX was associated with a decrease of total cell recruitment compared to primary infection. Regarding number of eosinophils, the number recovered from DEC-TPX immunized animals was significantly decreased when compared to the primary infection control, and it was decreased when compared to the pcDNA-TPX group. The neutrophils showed a different profile, with higher levels in pcDNA-TPX immunized mice than in either DEC-TPX vaccinated mice or a primary infection. The number of macrophages was significantly decreased in DEC-TPX vaccinated mice compared to a primary infection (Fig. 4.21).

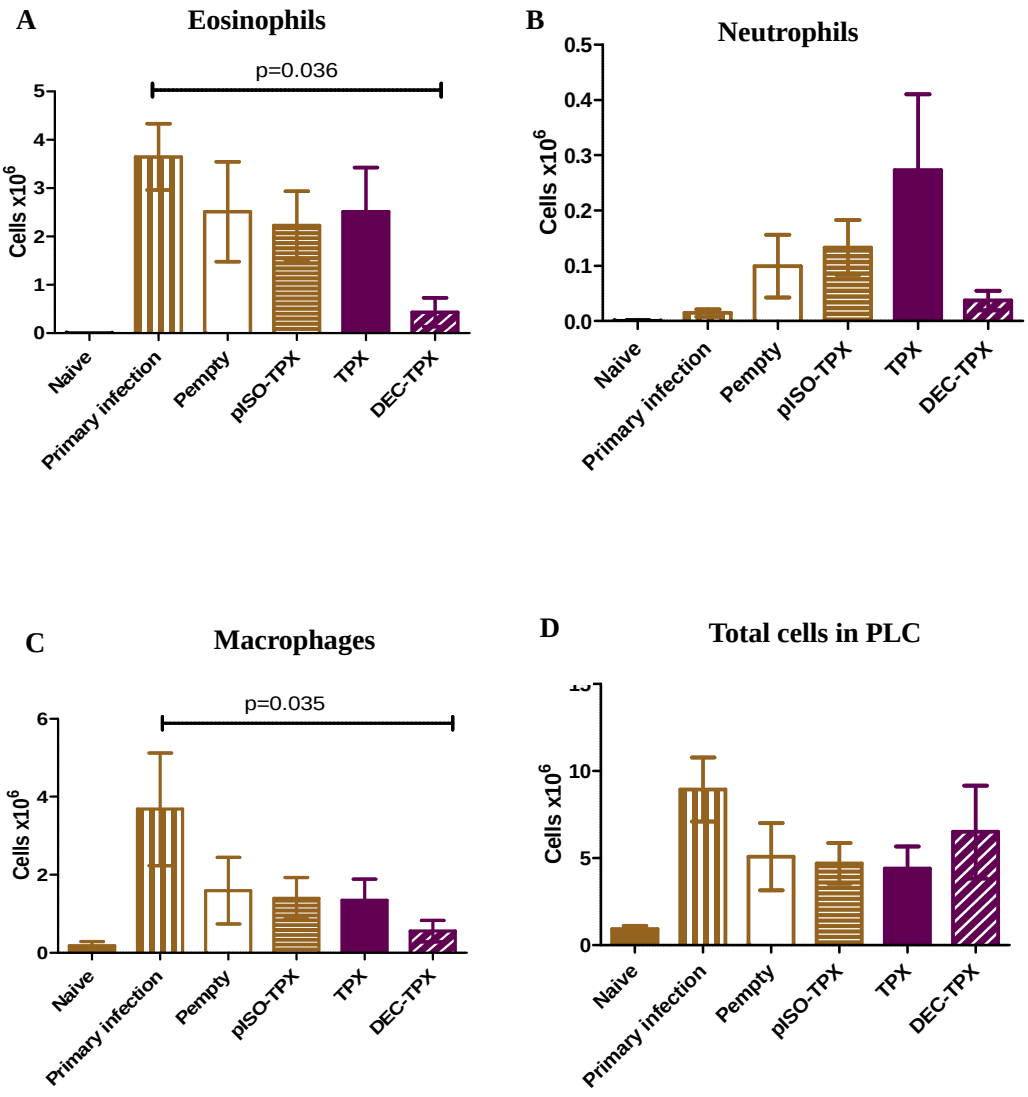


Fig. 4.21 Eosinophil, macrophage and neutrophil numbers were reduced following immunization with DEC-TPX when compared to levels recorded in a primary infection. Total cell numbers were determined using the CASY model TT cell counter system. Eosinophil, neutrophils and macrophage numbers were determined following concentration by cytospin and enumerated by microscopy on fixed slides (300 cells at a minimum on each slide were counted). 5 mice were tested in each group. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

4.2.19 Adult worm recovery was significantly decreased in mice vaccinated with DEC-TPX when compared to a primary infection

To evaluate the efficacy of the DEC-TPX vaccination, the number of worms recovered from the pleural cavity, and Mf in blood were determined on day 60 post challenge with 25 L3 larvae. Protection is defined as a reduction in worm number in test animals compared to those in control groups. As presented in Fig.4.22, the number of worms recovered from DEC-TPX vaccination was significantly lower than that recovered from mice with a primary infection ($p=0.0061$).

However, there was no difference between the numbers of worms recovered from mice vaccinated with DEC-TPX and those immunized with the pempty plasmid, and the pISO-TPX control and TPX alone.

As for the number of Mf recovered in the blood, there was an apparent reduction in numbers following vaccination with pcDNA-TPX and DEC-TPX, and DEC-TPX vaccination resulted in a statistically significant reduction of microfilariaemia.

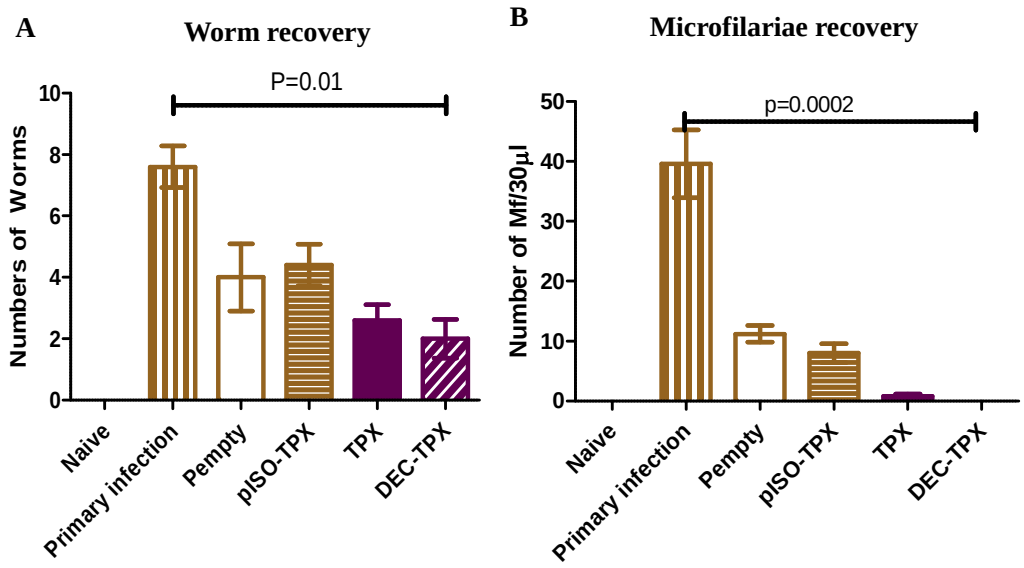


Fig.4.22. Adult worm recovery was significantly decreased in mice vaccinated with DEC-TPX when compared to a primary infection. Necropsy was performed 60 day post challenge, pleural cavity lavage was collected and worms were fixed with 70% hot ethanol; 30µl blood were added into 270µl FACS lysing solution then microfilariae were counted under microscope as well as the adult worms. The Mann-Whitney test was used as statistical method. P<0.05 represents a significant difference. Results are shown as the mean of replicate samples (+/- S.E.M). 5 mice were tested in each group.

4.3 Discussion

4.3.1 Ls-VAH and Ls-TPX as candidates for a filarial DNA vaccine

VAH was originally described as an abundantly expressed transcript of the L3 stage of filariae (*B. malayi*, Murray J *et al.*, 2001). The present work with *L. sigmodontis* showed that Ls-VAH is expressed by adult worms. Moreover, works by others (Schallig HD, van Leeuwen MA, 1997; Ghosh K *et al.*, 1996; Sen L *et al.*, 2000) have shown that mice can be protected from challenge infection with other parasitic nematodes by immunization with a recombinant VAH protein. For this reason, this molecule was chosen as a candidate for an experimental DNA vaccine.

In the case of Ls-TPX, the presented data revealed that it is expressed in the adult stage, although precisely how much is produced is not yet known. Interestingly, work with Ov-TPX2, suggested that expression of the gene declined with age and might reflect the decline in growth, basal metabolic rate and embryogenesis in adult worms (Karam M *et al.*, 1987; Strote G *et al.*, 1993). This may reflect the relative invulnerability of mature adults to immune attacks. TPX may be considered as primarily a housekeeping antioxidant and has been adapted to play a role in defense against the host immune response by protecting the parasites from damage by host-generated oxidative stress. For this reason, this molecule is considered a vaccine candidate.

4.3.2 Immunization with Ls-VAH and Ls-TPX induces a predominant antibody response

Human studies by Anand SB *et al* (2007) showed that the predominant antibody response of EN individuals against *W. bancrofti*-VAH was of the IgG1 and IgG2 isotypes with IgG3 being expressed at a lower level. However in infected individuals (Mf and chronic patients) this order was reversed with IgG3 antibodies being the predominant response followed by IgG2 and IgG1 isotypes. These observations were different with the previously reported isotype profiles seen with either Bm-VAH or Ov-VAH (Murray J *et al.,* 2001; MacDonald AJ *et al*., 2004) in which the most predominant isotype in protective individuals were IgG3 followed by IgG4 with very little IgG1 or IgG2 isotype.

Vaccination of unexposed adult volunteers with recombinant *Ancylostoma* secreted protein-2 (Na-ASP-2), a homologue of VAH, resulted in a predominant IgG1 response followed by IgG4 and IgE. However, no IgG2a or IgG3 antibodies were produced (Bethonya JM *et al.*, 2008).

These various observations suggest a strong Th2 response following natural infection and vaccination. The results presented with respect to *L. sigmodontis* showed the same trend,

although Ls-TPX stimulated a predominant IgG1 and IgE response, while Ls-VAH also induced IgG2a indicating a Th1 component in the response.

4.3.3 Vaccination with Ls-VAH not Ls-TPX induces a Th2-bias in cytokine responses

One of the aims of present study was to enhance the Th2-biased cytokines because of the association with the reduction in the number of worms recovered. Presented data showed DEC-VAH immunization significantly improved the expression of IL4, IL5 and IL13 in the pleural cavity 60 days post the *L. sigmodontis* challenge. DEC-TPX vaccination induced a similar trend of cytokines, as despite of the lack of IL4 production for DEC-TPX vaccination, IL5 and IL13 were much higher than controls but the IFNγ was also significant higher than TPX. These findings are important for present DNA vaccines because of the high similarities with responses to irradiated L3 vaccinations. This is important because irradiated L3 vaccines would not be acceptable for human use and DNA vaccines may provide an acceptable alternative.

4.3.4 IL10 should be taken into consideration when develop a DNA vaccine

One of the most important developments in the field of filarial immunity has been the recognition of regulatory networks in which Treg cells and IL10 play a predominant role. In humans, it has been shown that reduced levels of IL10 can, in part, explain the different clinical and parasitological manifestation of filarial infections (Specht S *et al*, 2010; Simons JE *et al*, 2010). This indicates that very careful attention should be paid to the production of IL10 by a vaccine: A low level of IL10 induction following vaccination may help to increase the protective response because of the lack of suppression associated with IL10.

Presented data showed that levels of IL10 were decreased in DEC-VAH vaccination with a

significant decrease following immunization with DEC-VAH compared to primary infection and pempty groups. This may be an indication that suppression induced by IL10 does not impact the protective responses induced by DEC-VAH immunization, and provide an explanation of enhanced immune responses with immunization of DEC-VAH. This speculation is supported by result of undetectable IL10 in mice immunized with combination vaccine (DEC-ADDLAT and DEC-CPImu, chapter 3), which eventually achieved a high protection.

4.3.5 Factors associated with protective responses induced by single DEC-VAH or DEC-TPX vaccination

Presented data showed that the number of worms recovered after DEC-VAH immunization was lower than that of the primary infection group, while the number of worms recovered after DEC-TPX vaccination was significantly lower than that of primary infection ($p=0.011$). However, in the case of DEC-TPX vaccination, when the worm burden of mice immunized in control DNA vaccines was measured, there was no difference between control and test groups. This result must be investigated further. It should be remembered that the number of worms recovered from immunized mice and the number of challenge L3 given are small. This makes identification of statistical significantly differences very difficult. Much larger experimental groups of mice must be used to overcome this problem.

A second consideration may be the complicated life cycle of filarial nematode. The possibility must be considered that the effective vaccination may require one or more antigens must be targeted to different life stages. To take VAH as an example, Bethony JM *et al* (2008) found that vaccination with recombinant Na-ASP-2 (a homologue of VAH) could not prevent L3 from reaching the gastrointestinal tract and developing into blood-feeding adult worms.

Additionally, attention must be paid to a possible role for Th1 responses in protection. One

of the major advantages of DNA vaccines is that they can induce Th1 as well as Th2 responses. Indeed, research in experimental parasite systems suggests a role of Th1 in protective responses (Saeftel M *et al.,* 2001; Turaga PS *et al.,* 2000; Anand SB *et al.,* 2007; MacDonald AJ *et al.*,2004; Dabir P *et al.,* 2006). This indicates that a Th1 response might be important for VAH vaccination to induce protection. In *W. bancrofti,* the Wb-VAH primarily induced a Th1 type response on EN individuals. Similar responses occurred to Bm-VAH-1. In the case of DEC-VAH, it induced high levels of IgG2a and decreased IFNγ, which might imply that for this VAH, Th1-biased responses might be more important than Th2 responses. However, this speculation has not been investigated in the present work.

Although it is clear that protein vaccines can induce protection (Schallig HD *et al.,* 1997; Ghosh K *et al*., 1996; Sen L *et al.*, 2000), it is difficult to conclude that protein vaccines are better than DNA vaccines. Rather, it is still necessary to conduct detailed investigation of both approaches. Nevertheless, the work presented with the *L. sigmodontis* model and immunization with a combination DNA vaccine (chapter 3) did show a good protective response.

Chapter 5-Partial protection has been achieved by ADDALT, CPImu and VAH or TPX cocktail vaccination

5.1 Introduction

DNA vaccine has been shown to be an effective way to produce protective immune responses against a wide range of viral and bacterial pathogens (Chang SW *et al.*, 1998; Fynan EF *et al.*, 1993; Lowrie DB *et al.*, 1999; Strugnell RA *et al.*, 1997) and more recently in eukaryotes including malaria (Hill AV *et al.,* 2010) and gastrointestinal parasites (Rothel JS *et al.*, 1997). However, the complex parasite life cycles, involving the stage-specific antigens expession, numbers of different anatomical and immunological compartments in their hosts, and the abilities of modulation of their host's immune responses, suggest an effective vaccine may necessarily comprise a number of different antigens.

The possibility of combining a number of plasmids in a cocktail vaccine could be very attractive. This possibility has been explored by Zhang YB *et al* (2002) using a cocktail DNA vaccine comprising 4 DNA plasmids which encode 4 different *S. japonicum* antigens, Sj62, Sj28, Sj23 and Sj14-3-3, respectively. A significant cellular response with a high production of IFNγ in splenocytes was induced by recombinant antigens stimulation *in vitro* following three i.m injections of the cocktail DNA vaccine. Significant resistance (34-37 for single DNA vaccine and 44-45% for cocktail vaccine, respectively) against *S. japonicum* cercarial challenge has also been reported. Fachado A *et al* (2003) described a long-lasting protection induced by a DNA cocktail immunization against a lethal challenge infection with the virulent *T. gondii* RH strain, whereas low doses of single vaccine were not protective. Finally, Anand SB *et al* (2008) evaluated the protective efficacy and cell-mediated immune response a cocktail vaccine encoding Bm-ALT-2 and Bm-TPX in mice. Results showed that mice treated with a cocktail vaccine induced 78% of cytotoxicity against *B. malayi* Mf, but the single vaccine induced only 37%. Taken together, the combination of two or more

antigens may be an effective vaccine development strategy to improve protection and immunogenicity against filariasis.

The results presented in chapter 3 & 4 showed that co-vaccination with ADDALT and CPImu (in association with single chain antibody DEC205 targeting to the surface receptor DEC205 of DCs) can achieve significant reduction of worm and Mf burden, whereas the immunization of ADDALT alone or CPImu alone could not induce protection. The other two antigens, VAH and TPX, could induce Th2-biased immune responses with DEC205, however they did not afford any protection. These results suggested an opportunity to test the hypothesis that a combination of antigens/ plasmids in a single vaccine may potentiate responses against individual components. In addition, the observation that the DEC205 antibody was crucial for stimulation of enhanced responses also indicated that the choice of adjuvant will also be critical.

The work demonstrated below aims to induce high immunities with variable combinations of ADDALT, CPImu, VAH and/ or TPX with adjuvants of IL4 or IL4 plus MIP1α and Flt3L.

5.2 Results

5.2.1 Bi- and Tri- rather than Tetra-vaccine induced higher production of IgG1 compared to backbone plasmid

Four vaccines comprising DEC-ADDALT alone (designated Single-vaccine), DEC-ADDALT plus DEC-CPImu (designated Bi-vaccine), DEC-ADDALT plus DEC-CPImu plus DEC-VAH (designated Tri-vaccine), and DEC-ADDALT plus DEC-CPImu plus DEC-VAH plus DEC-TPX (designated Tetra-vaccine) were compared to a primary infection. As a control, a group of mice was also immunized with the carrier plasmid (pempty) that did not contain any parasite gene. All mice were given the same total volume and concentration of total DNA irrespective of whether they were immunized with a

recombinant alone or with a plasmid encoding IL4 as adjuvant. Mice were given two immunizations separated by two weeks and challenged with 25 L3s one month after the final immunization. Necropsy was performed and sera, pleural cavity lavage, blood for Mf and lymph nodes were harvested 60 days after challenge. As displayed in Fig. 5.1, the Tri-vaccine stimulated the strongest Th2 response with the highest levels of IgG1 and IgE. This together with the Bi-vaccine revealed that significant elevation of IgG1 levels over the control groups. The Single-vaccine did however stimulate a stronger and statistically significant IgG2a [Th1] response than pempty and primary infection. The apparent enhanced Th2 biased response by Bi- and Tri-vaccines was however sharply reversed by the results obtained with the Tetra-vaccine, which failed to induce any responses over and above those measured in the controls.

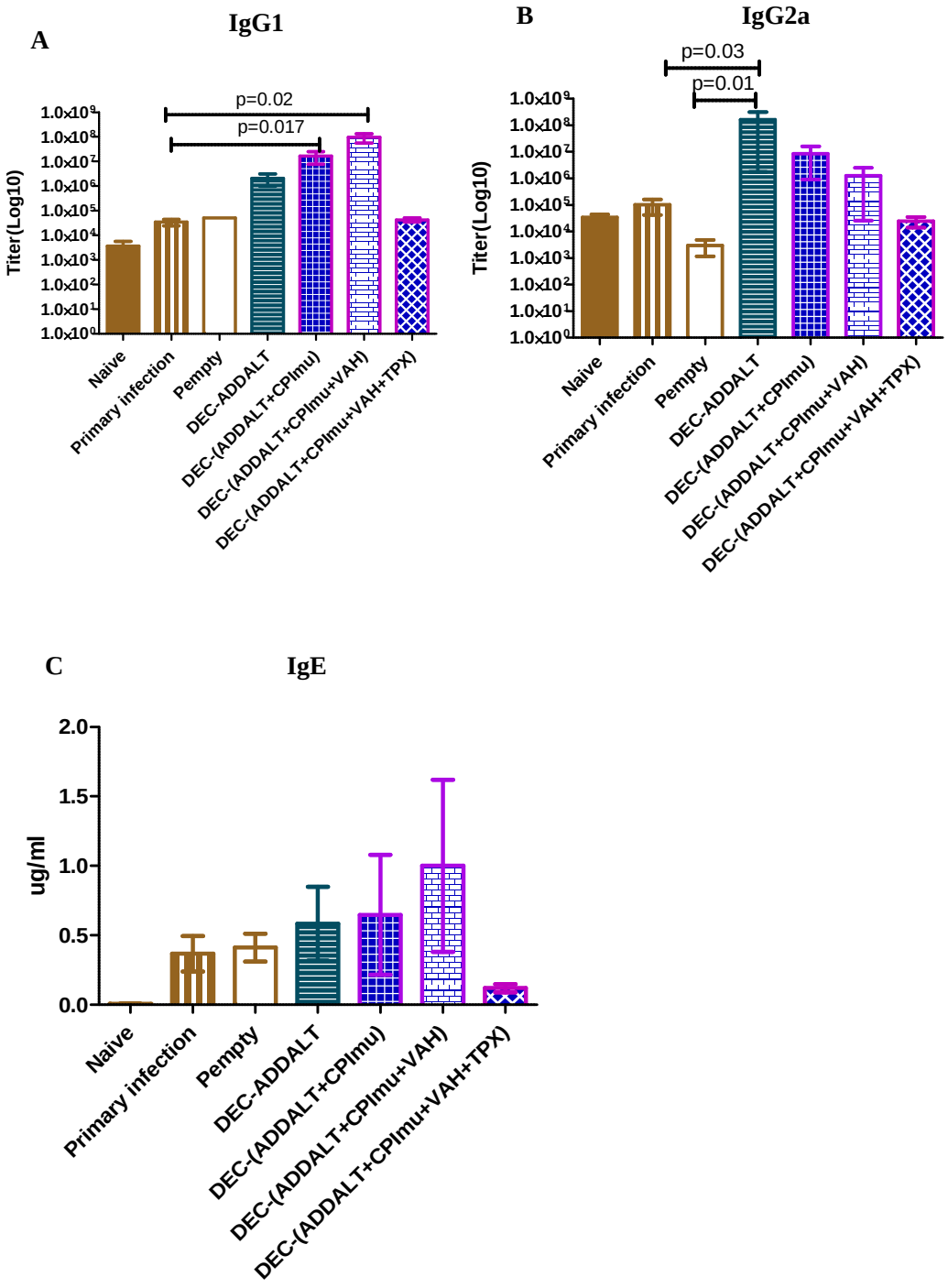
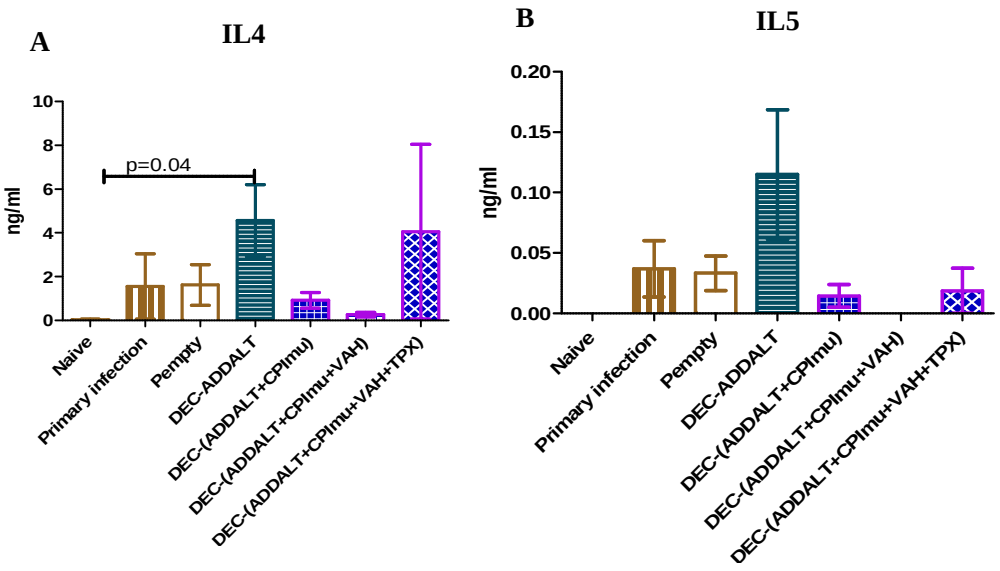


Fig. 5.1 Antibody responses induced by cocktail vaccinations. Antibody levels of IgG and IgE were compared between Single-, Bi-, Tri- and Tetra-vaccine by indirect ELISA. All test groups were included adjuvants IL4. Results are shown as titer of IgG and the mean of replicate samples (+/- S.E.M) for total IgE. Mann-Whitney test was used as statistical method. Differences were considered significant for $p < 0.05$; p values were not reported otherwise. 5 mice per group were tested.

5.2.2 No significant difference on cytokine production by vaccinations

To determine the cellular responses provoked by cocktail vaccinations, the levels of cytokine in the pleural cavity were measured by capture ELISA. As shown in Fig.5.2. D, slightly higher IL13 was induced by Single- and Bi-vaccines compared to backbone control. An increased level of IL13 was induced by Tri-vaccination in contrast to the Single- and Bi-vaccines. Although a significant increase in IL13 was produced by Tri-vaccination when compared to primary infection, there was no significant difference between Tri-, Bi-, and Single-vaccine. Further, the Tetra-vaccine did not induce IL13 production. Although it was possible to detect IL4, IL5 and IL10, there was no statistically significant difference between test and control groups. Similar results between groups were seen with respective to the Th1 associated IFNγ.



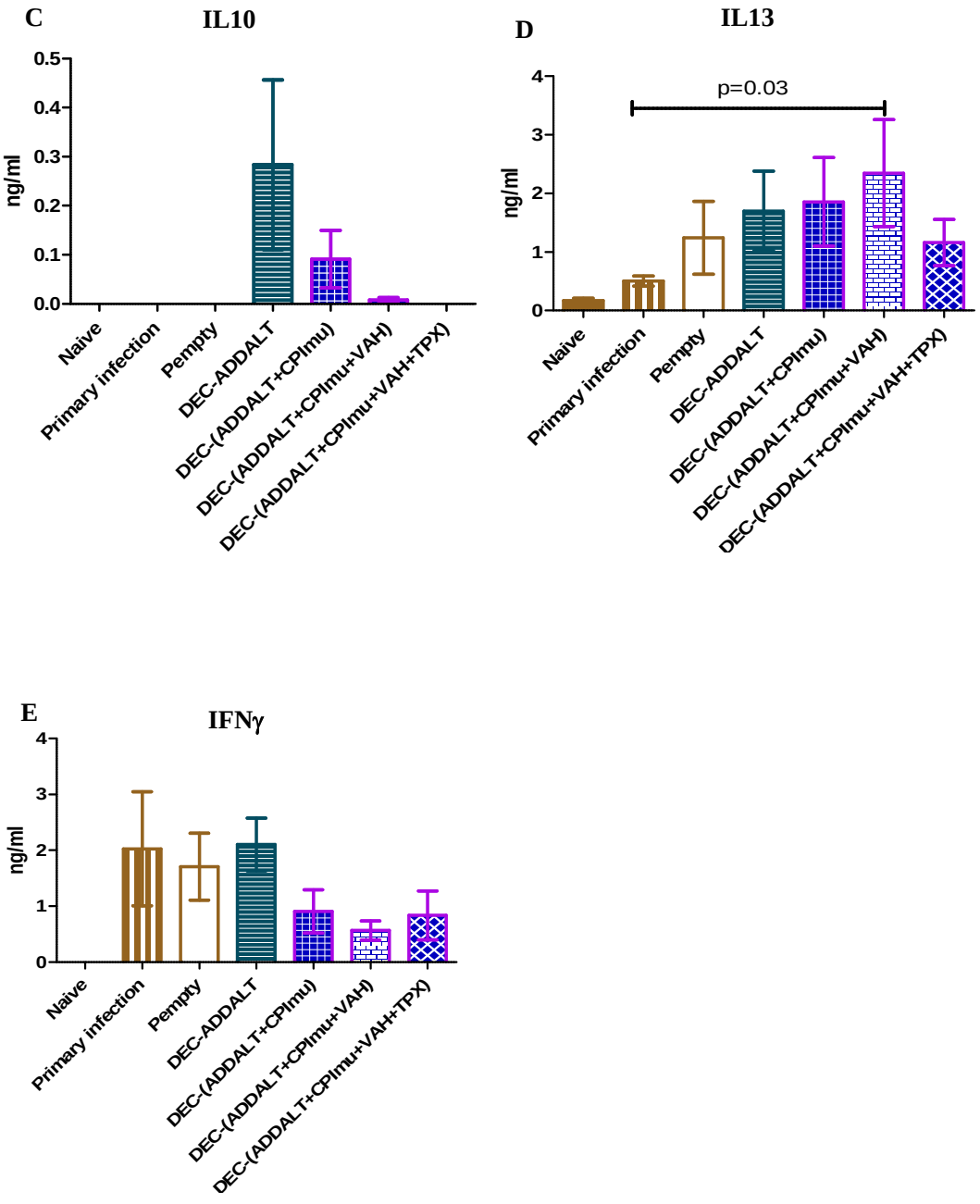


Fig.5.2 Cytokine production in pleural cavity lavage induced by cocktail vaccinations. Supernatant of pleural cavity lavage were harvested and the levels of various cytokines (IL4, IL5, IL13, IL10 and IFNγ) were measured by sandwich ELISA-(see methods and materials). Results are shown as the mean of replicate samples. 5 mice per group were tested. . The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyze differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise

5.2.3 Significantly higher numbers of eosinophils were recruited in the pleural cavity by Tri- and Tetra-vaccinations

To determine the numbers and types of cells recruited in the pleural cavity where adult worms live, the pleural cavity was lavaged and cells recruited were counted via the cytospin assay. Cell numbers were counted under a microscope. As shown in Fig. 5.3 A, compared to pempty control, eosinophil numbers were significantly elevated by vaccination with Tri- and Tetra-vaccines compared to the primary infection group, although the number in the Tetra-vaccine group was lower than that in the Tri-vaccine group.

5.2.4 Cocktail vaccines induced significantly higher number of macrophages but not neutrophils compared to the Single-vaccine

Since macrophages and neutrophils are primary defense against pathogens in the innate immune system, the numbers of these cells were measured in vaccinated mice following L3 challenge. Neutrophil numbers were significantly increased in mice receiving the Single-vaccine over and above those measured in control groups. Although apparent numbers of neutrophils were also seen following immunization with Bi-, Tri- and Tetra-vaccine, the numbers were lower than the Single-vaccine and there was no statistical difference between these groups and controls (Fig. 5.3).

Interestingly, macrophage numbers in mice immunized with pempty and in the animals with a primary infection were significantly higher than in naïve mice, Bi-, Tri- and Tetra-vaccine. At this stage, no explanation can be given with regard to this observation (Fig. 5.3).

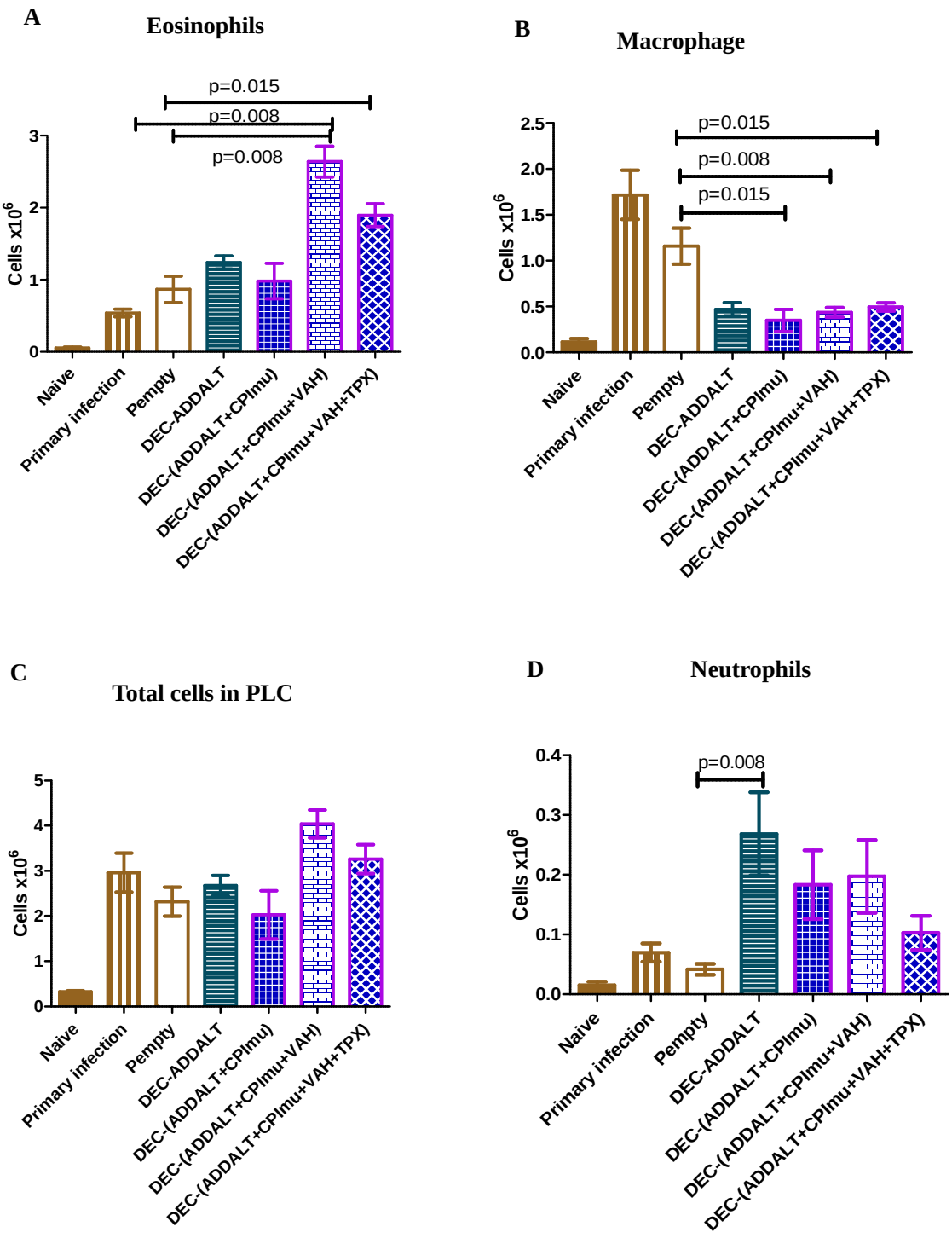


Fig.5.3 Cells recruited in pleural cavity by cocktail vaccinations. Total cell numbers were determined using the CASY model TT cell counter system. Eosinophil, neutrophils and macrophage numbers were determined following concentration by cytospin and enumerated by microscopy on fixed slides (300 cells at a minimum on each slide were counted). The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyse differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise. 5 mice per group were tested

5.2.5 Significant reduction of worm burden but not microfilariae numbers was achieved by the Tri-vaccine immunization

To evaluate the protective response elicited by the cocktail vaccinations, worms in the pleural cavity and Mf in the blood stream were counted. A challenge of 25 L3s were given to each mouse, and worms recovered at day 60 post challenge were counted. Protection was recorded as a reduction in worm numbers in test animals compared to those in control groups. As presented in Fig.5.4. A, the number of worms recovered from Tri-vaccine immunization was significantly lower than that in the primary infection group, showing that protection was obtained by the Tri-vaccine. However, the Tetra-vaccine did not provide protection. The Single-and Bi-vaccine did not result in the reduction of worm burden. As for the recovery of Mf, the number varied among groups and no significant differences were found between test groups and controls.

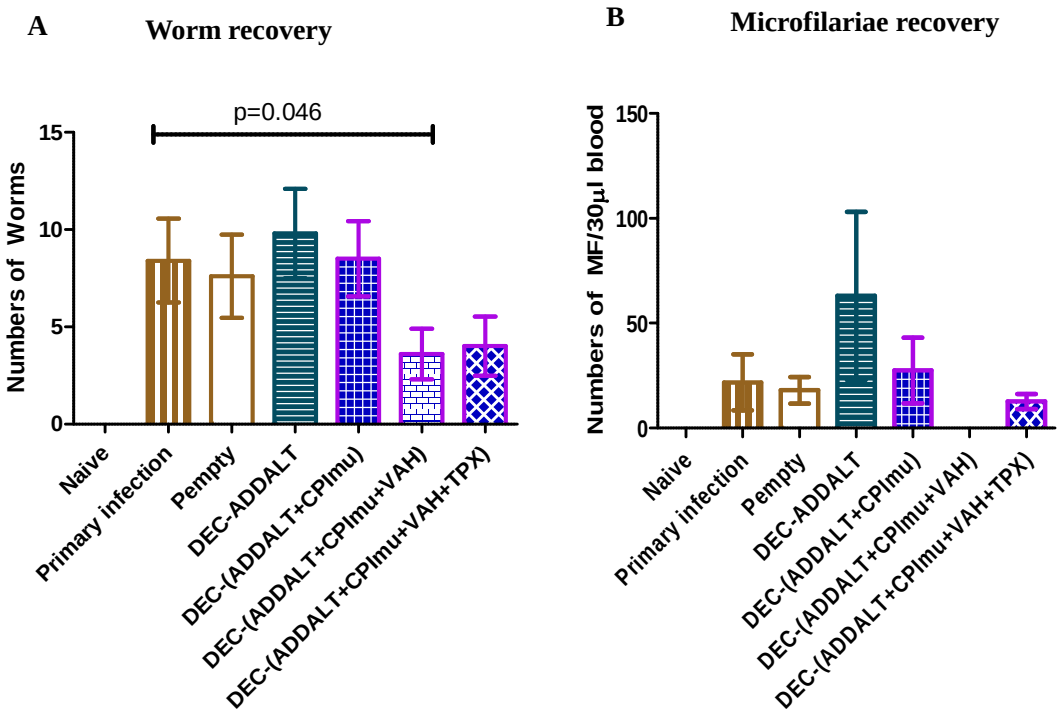


Fig.5.4 A significant reduction in adult worm rather than microfilariae was achieved by Tri-vaccine immunization. Necropsy was performed 60 day post challenge, pleural cavity lavage was collected and worms were fixed with 70% hot ethanol; 30 µl blood was added into 270µl FACS lysing solution then microfilariae were counted under a microscope as well as adult worms. Mann-Whitney test was used as a statistical method. Differences were considered significant for $p < 0.05$; p values were not reported otherwise. 5 mice per group were tested.

5.2.6 Both Tri-VAH and Tri-TPX induced protective responses

Results in chapter 3 together with the presented IgG in Fig 5.1 and Fig. 5.5, suggested that ADDALT and CPImu may provide a core component of a vaccine where immunogenicity and protective responses can be enhanced by addition of other antigens such as VAH and TPX. To test this idea further, several experiments were designed to compare VAH and TPX as vaccines in their own right and in company with ADDALT and CPImu. Six vaccination strategies comprising of DEC-VAH alone, DEC-TPX alone, DEC-VAH plus DEC-ADDALT plus DEC-CPImu (designated Tri-VAH), DEC-TPX plus DEC-ADDALT plus DEC-CPImu (designated Tri-TPX), backbone plasmid (pempty) and primary infection were set up. All mice were given the same total volume of vaccine containing equivalent

amounts of DNA irrespective of the recombinant formulation. Mice were also inoculated with plasmids encoding IL4, MIP1α and Flt3L as adjuvants. Mice were given two immunizations separated by two weeks and challenged with 30 L3s one month after the final immunization. Necropsy was performed and sera, pleural cavity lavage, blood for Mf and lymph nodes were harvested 60 days after challenge.

As shown in Fig 5.5, the Tri-VAH vaccine again induced significantly higher levels of IgG1 when compared to control groups. Similarly, the Tri-TPX in which TPX replaced VAH, also induced significantly levels of IgG1. However, VAH or TPX alone did not stimulate any responses over and above controls.

Although levels of IgE and IgG2a were detected, there was no significant difference between test groups and controls.

When IL4, IL5, IL13, IL10 and IFNγ were measured, no significant differences were detected between test and control groups (Fig. 5.6). Nor there was any difference in recruitment of eosinophils, neutrophils and macrophages in the pleural cavity in mice (Fig. 5.7).

However, as shown in Fig. 5.8, vaccination with Tri-VAH and Tri-TPX did result in significant reductions in worm burden (55.7% and 41.6%, respectively). In addition, vaccination with Tri-TPX also resulted in a significant reduction in Mf.

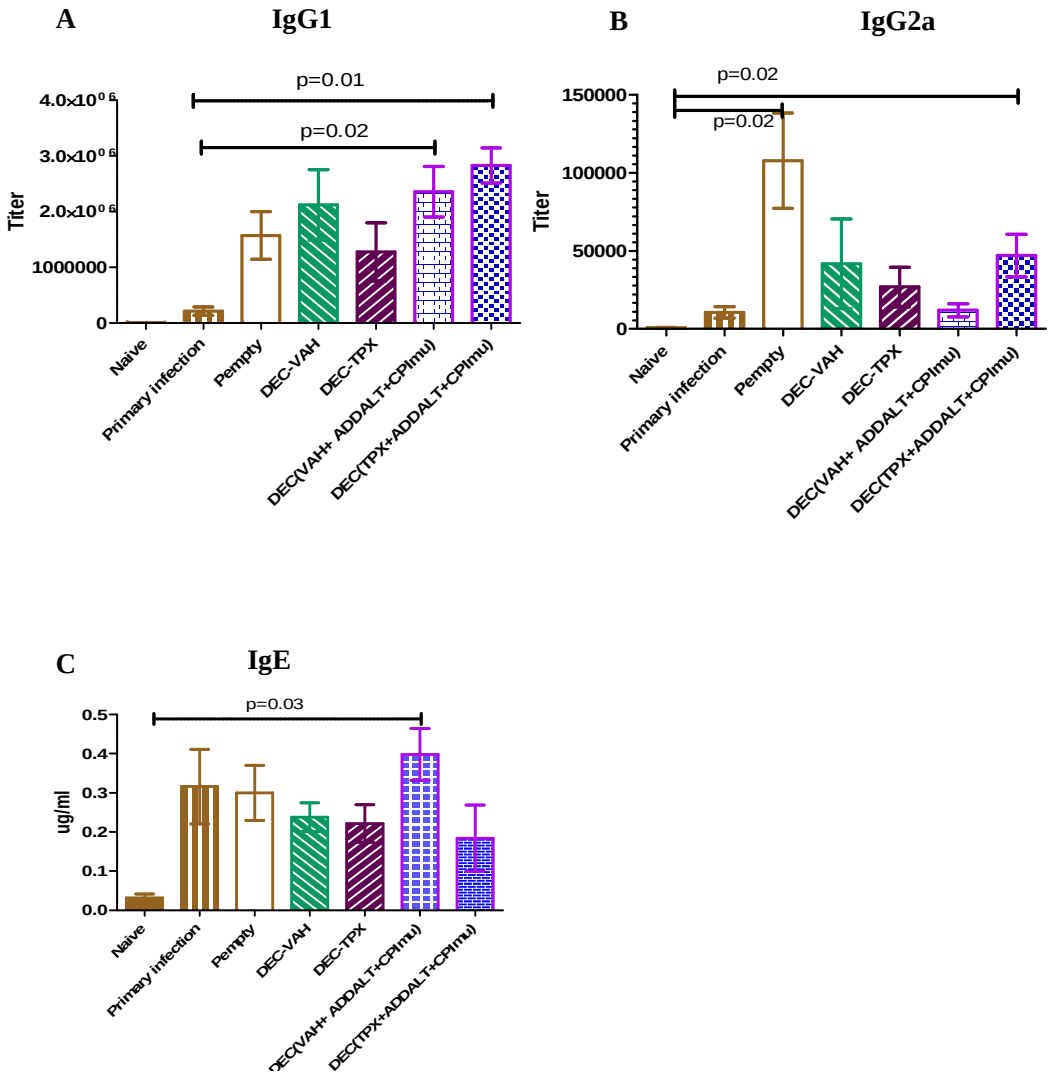


Fig. 5. 5 Antibody responses induced by cocktail vaccinations. Antibody levels of IgG and IgE were compared by indirect ELISA. All test groups included the adjuvants IL4, Flt3L and MIP1α. Results are shown as titer of IgG and the mean of replicate samples (+/- S.E.M) for total IgE. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyse differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.5 mice per group were tested.

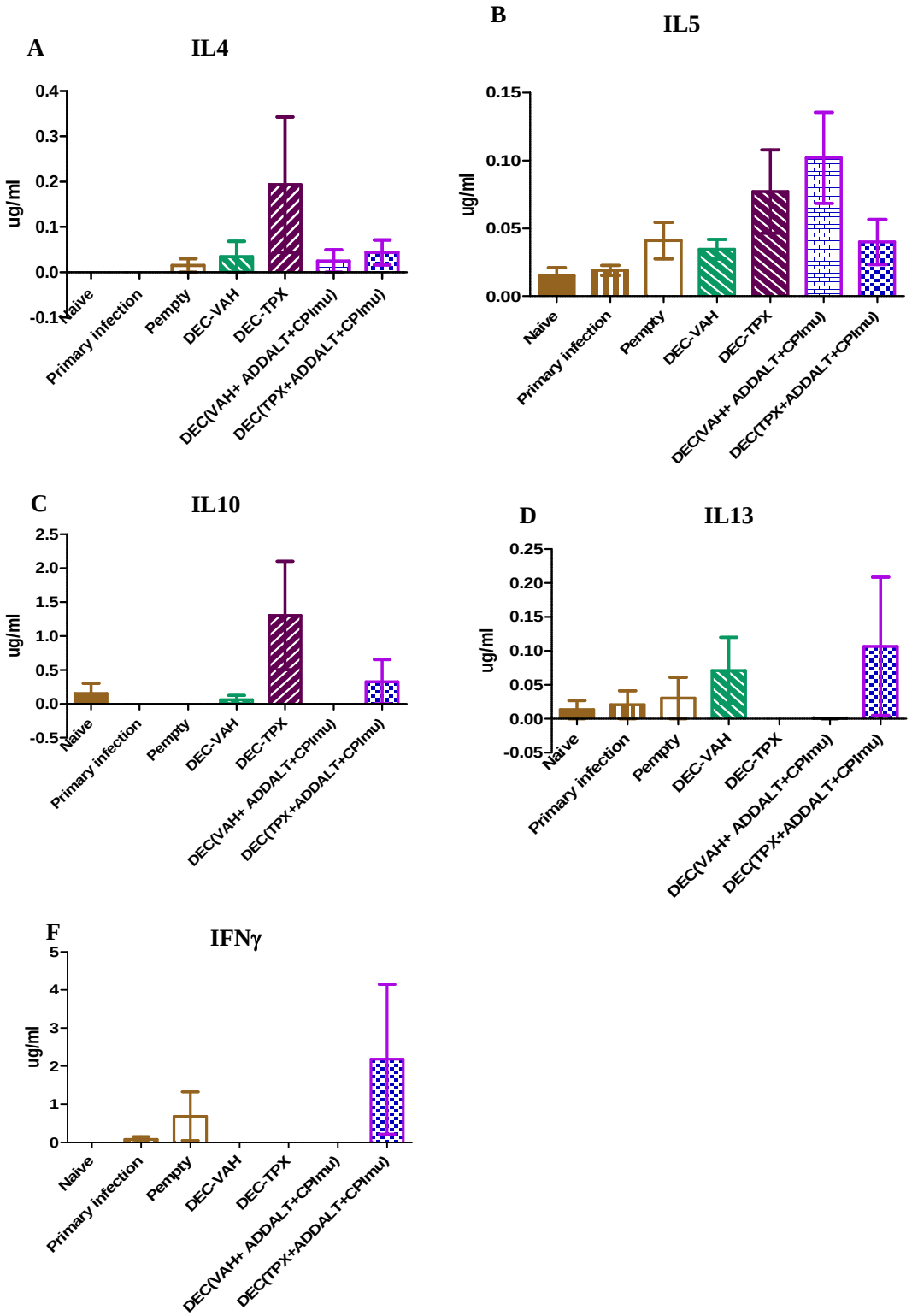


Fig.5.6 Cytokine production induced by cocktail vaccinations in the pleural cavity lavage fluid. Supernatant of pleural cavity lavage were harvested and the level of various cytokines (IL4, IL5, IL13, IL10 and IFNγ) were measured by capture ELISA- (see methods and materials). Results are shown as the mean of replicate samples. 5 mice per group were used. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyse differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise

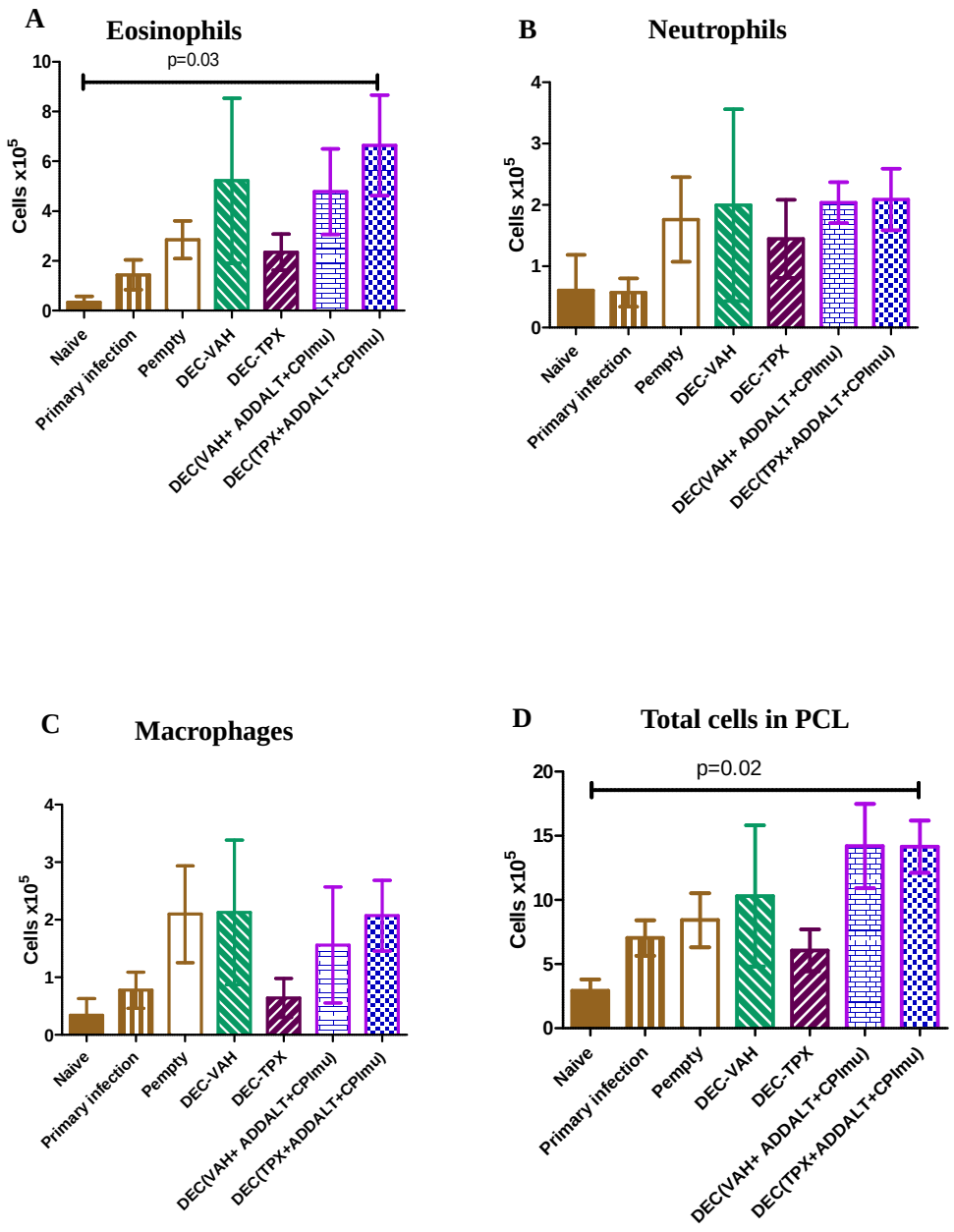


Fig.5.7 Cells recruited by cocktail vaccinations in the pleural cavity. Total cell numbers were determined using the CASY model TT cell counter system. Eosinophil, neutrophil and macrophage numbers were determined following concentration by cytospin and enumerated by microscopy on fixed slides (300 cells at a minimum on each slide were counted). 5 mice per group were used. The Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyse differences between groups. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.

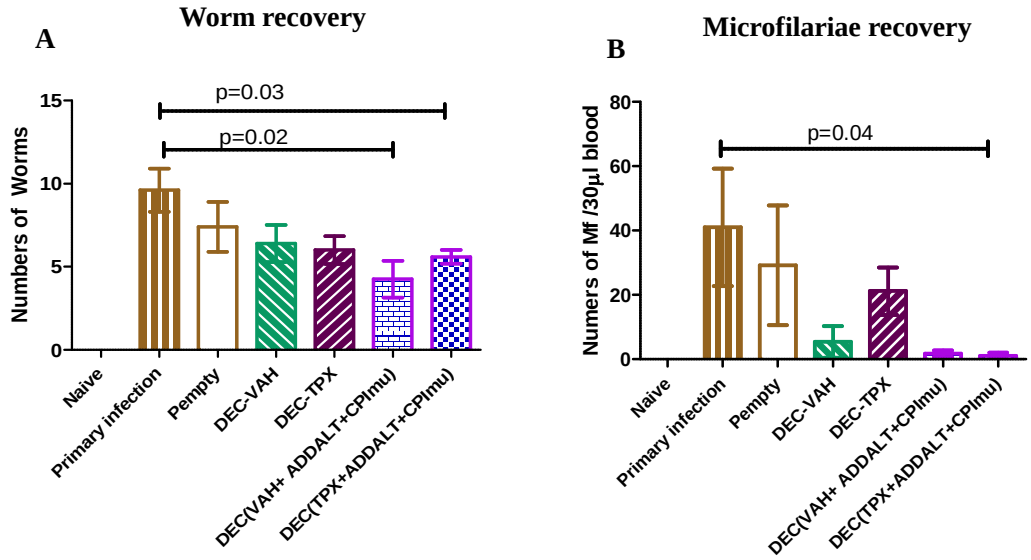


Fig.5.8 Significant reduction of adult worm and microfilariae burden were achieved by cocktail immunizations. Necropsy was performed 60 day post challenge, pleural cavity lavage was collected and worms were fixed with 70% hot ethanol; 30 µl blood were added into 270µl FACS lysing solution then microfilariae were counted under a microscope as well as adult worms. Mann-Whitney test was used as a statistical method. Differences were considered significant for $p < 0.05$; p values were not reported otherwise.5 mice per group were used.

5.3 Discussion

5.3.1 Cocktail vaccination is a promising approach for a more efficacious vaccine against filarial infection

The goal of immunization is the induction of a protective immune response against the pathogen. In the case of filarial nematodes, the complex life cycle presents a plurality of antigenic epitopes whose expression can vary widely between each developmental stage. In this situation, a broad immunity targeting several different antigens may be required to produce a more efficacious vaccine. Presented data showed that partial protection can be achieved through immunization with plasmids carrying Tri-VAH (55.7%) and Tri-TPX

(41.6%), while the single vaccines DEC-VAH and DEC-TPX obtained no protections, suggesting cocktail vaccines might be the better strategy to stimulate the host immune system to fight against filarial infection. This was also supported by the findings in chapter 3 in which the combination of DEC-ADDALT and DEC-CPImu induced stronger humoral and cellular immune responses as well as a significantly lower worm burden than the single DEC-ADDALT or DEC-CPImu vaccines. Similar reduction of worm burden has been judged on vaccination with four *S. japonicum* antigens (Zhang Y *et al*, 2001). In this sense, it appears that DNA cocktail can induce more efficacious protective responses than a single recombinant plasmid, although present work needs more investigation.

5.3.2 Ratio and quantity of components in cocktail vaccine may impact protective responses

DNA cocktail vaccines can induce stronger protective responses than a single vaccine (Anand SB *et al.*, 2008; Jongert E *et al.*, 2007; Ahmed SB *et al.*, 2009; Park SU *et al.*, 2008; Williamson ED *et al*., 2002). Presented data suggested that the immune responses and the protection were associated with the relative amount of an individual component. Results from the experiment that designed to compare the effects of Single-, Bi-, Tri- and Tetra-vaccines showed that the best immune response and significant reduction of worm burden were only observed with the Tri-vaccine but not the Single-, Bi- nor Tetra-vaccines. The reason might be the lower amount of an individual ingredient in the Tetra-vaccine compared with the others. The best ratio of individual components in a cocktail vaccine might be 1:1:1. This was supported by a subsequent experiment in which the cocktail vaccines were composed only by three individual ingredients (the ratio was 1:1:1) with the same amount of each component. This immunization led to the protective responses with Tri-TPX vaccination, in which the amount of TPX had been increased in contrast to the previous lower amount of TPX in Tetra-vaccination.

However, what has not yet been tested is whether variation of the amount of individual

components (ie. a dose response) may influence responses with respect to the determinant responses and protective immunity. Second, the optimal quantity of individual component must be investigated. Because, Fachado A *et al* (2003) showed that injection of 25 and 50 μg of DNA cocktail vaccine against *T. gondii* infection (containing 12.5 or 25 μg per plasmid) can induce both humoral and cellular immune responses which cannot be elicited by equivalent doses of a single vaccine, respectively. Third, the quantity of adjuvant requires more investigation. This experiment has used DEC205 to target DCs, but more conventional adjuvants, such as alum, may also influence responses against the target antigens.

5.3.3 Cytokine production of cocktail vaccination

Presented data showed that levels of cytokines were rarely enhanced following cocktail immunization, only increased IL13 (but not IL4, IL5 and IL10) was induced by the Tri-vaccine (but not Bi- nor Tetra-vaccine). Interestingly, significantly increased IL13 was found in mice immunized with a combined vaccine (chapter 3) which may be an indication that individual components can be critical to cytokine production. Again, this relates to formulation of the vaccine, but also important was the absence of induction of IL10 which has the potential to modulate vaccine-mediated protective immunity (discussed in chapter 3, 4).

Chapter 6- General discussion

6.1 Summary of experimental results

Four candidates (ADDALT, CPImu, VAH and TPX) as DNA vaccines against filarial infection were tested in *L. sigmodontis*-BALB/c mouse model. ADDALT and CPImu were engineered variants of ALT and CPI from which sequences encoding peptides that have immunosuppressive functions have been removed. In an attempt to enhance protective responses, the ability to target antigen presentation cell (DCs) was used. For this purpose, combinations of plasmids encoding single chain antibody with specificity of DEC205 were tested. Humoral responses (IgG1, IgG2a, IgE), cellular responses (cytokines IL4, IL5, IL10, IL13 and IFNγ), cells recruited in pleural cavity (eosinophils, neutrophils, macrophages, lymphocytes/ monocytes) and protection (adult worms and Mf recovery) have been investigated to assess the effects of vaccinations.

Immunization with these individual plasmids enhanced production of IgG1 and IgE, Th2 cytokines and increased eosinophils while levels of IgG2a and IFNγ were decreased. However, this bias towards a Th2 response did not result in any significant reduction of worm burden or microfilariaemia.

When a cocktail vaccine comprising ADDALT plus CPImu was used, both humoral and cellular responses were enhanced in contrast to the single antigen plasmid vaccines. More important, vaccination resulted in a significant reduction of worm burden (82.3%).

Subsequently, cocktail vaccinations composed of ADDALT, CPImu and VAH or TPX had demonstrated that Tri-VAH (ADDALT, CPImu and VAH) achieved 55.7% protection while Tri-TPX (ADDALT, CPImu and TPX) only obtained 41.6% reduction of worm burden.

6.2 Discussion

There are two ways of thinking about vaccination: 1), to mimic and enhance a response that is known to be protective following a natural infection; and 2), to design a vaccine that stimulates a protective response that is novel and not found following a natural infection, or may reverse or circumvent a response that would normally interfere or block a protective response.

The rationale design of a vaccine requires a thorough understanding of immune responses evoked by natural infection, both with respect to protection and possible pathology (allergic responses). In the case of filariae, the role of Th2 in protection against filarial infection was demonstrated by experiments with iL3s (Le Goff L *et al.*, 2000; Babayan SA *et al.*, 2006; Hübner MP *et al.*, 2010). Nevertheless, the core strategy of present vaccines is to enhance Th2-biased immune responses, which presents as an increased production of IgG1, IL4, IL5, IL13, and decreased IgG2a and IFNγ, although there is another voice that the synergism of both Th1 and Th2 responses might contribute to the successful protection (Saeftel M *et al.*, 2001). The experiments reported demonstrated significant reduction of worm burden following vaccination with a cocktail of DEC-ADDALT and DEC-CPImu. This vaccine induced high levels of IgG1, increased IL5 and IL13, the characteristic of a Th2 response. Although allergy can associate with Th2 response, it was not demonstrated in present study.

The protection afforded by ADDALT plus CPImu cocktail was produced through use of recombinant plasmid in DNA delivery system. It should be remembered that DNA vaccines can also stimulate a Th1 response. In the present study, strong Th1 responses were not demonstrated. At this stage, a contribution towards protection by Th1 responses cannot be ruled out.

For a successful vaccine, the most important inspection item is the high protection against infection. Current data indicated that the dual vaccine comprising ADDALT and CPImu

candidates resulted in 82.3% reduction of worm burden and the cocktail vaccine comprising ADDALT, CPImu and VAH achieved 55.7% protection against *L.sigmodontis* infection in BALB/c mouse model, suggesting current DNA vaccine formulas were successfully tested and might be a promising logical feasible way against filarial nematodes. For vaccine development against filarial nematodes including onchocerciasis, *W. bancrofti* and *B. malayi*, various strategies such as epitope, protein, radiation-attenuated L3, recombinant engineering vaccines have been investigated previously, but none of them (except iL3, some can reach 100% protection) obtained as high protection as present DNA vaccination did so far. For instance, two B epitopes from the antioxidant thioredoxin of *B. malayi* were synthesized to produce a single peptide conjugate (PC1) vaccine and this vaccine can produce a significantly high protection (75.14%) in experimental filariasis (Madhumathi J *et al.,* 2010). A mean protection of 75.86% was achieved by vaccination with *Setaria cervi* 175 kDa collagenase against *B. malayi* infection in jirds (Pokharel DR *et al.,* 2006). Immunization with iL3 against *L. sigmodontis* in mice obtained a long-term protection (54-58%) against the L3 larvae (Babayan SA *et al.,* 2006). Roughly 61% protection was achieved against a *B. malayi* when a recombinant GST from *W. Bancroft* was immunized in a jird model (Veerapathran A *et al.,* 2009) and a 50% reduction in larvae survival was obtained when recombinant *O. volvulus* glycolytic enzyme fructose-1,6-bisphosphate aldolase was tested in a mouse chamber model (McCarthy JS *et al.,* 2002). However, present works have been demonstrated to be profound with comparison to previous vaccines, even the same vaccine type (DNA vaccine) against filarial nematodes. Immunization with Bm-ALT DNA vaccine in jirds produced 57% protection (Thirugnanam S *et al.,* 2007), while DNA immunization with *O. volvulus* chitinase produced 53% protection against challenge infection with L3 larvae in mice (Makepeace BL *et al.,* 2009) and the cocktail vaccine comprising Bm-ALT2 and Bm-TPX induced only 78% of cytotoxicity against *B. malayi* Mf. In this sense, current dual vaccine might be the highest protection obtained by DNA vaccine against filarial nematodes so far. One might argue that it is the different natures of candidates that results in the diverse protections. General speaking, it is true that the characteristics of candidate antigens are

essential for vaccine development, which is the main reason why scientists keep seeking promising antigens to investigate the protective effects they may induce, however, it is not the only factor that have to be considered when develop a successful vaccine. Many factors are involved, for instance, the pre-dominant responses the vaccine should elicit, the main killing cells that vaccine should promote, the adaptive adjuvant that vaccine should use, and so on. Four antigens for vaccine development against filarial nematodes, ALT, CPI2, VAH and TPX have been suggested to be promise as vaccines candidates. However, seldom DNA vaccines comprising all these four antigens so far have been proved to be success, despite the protein vaccine of Bm-ALT2 conferred about 75% protection (Thirugnanam S *et al.*, 2007) and Bm-TPX in jirds exhibited 69% protection (Vanam U *et al.*, 2009). Present works selected all these four antigens to develop cocktail vaccines instead of single vaccine, results showed that high protection was obtained by dual vaccination, suggesting single antigen might not be enough to induce strong protective immune responses. In this sense, the individual nature of single antigen might not be the only important factor when developing cocktail vaccine, the importance might be the co-reaction of each antigen in cocktail vaccine regimen. Current works were carried out on the *L. sigmodontis/* mouse model, not jirds, gerbil nor cattle, which might be one of the reasons that high protection could be achieved. *L. sigmodontis/* mouse model provides an excellent model system to investigate the immune mechanisms and vaccine development because of the advantage of allowing the observation of the full life cycle of the parasite, including the development of L3 moulting to L4 and adult worms and the Mf stage circulating in the bloodstream in susceptible BALB/c mice. All these merits provided for filarial vaccine development by *L. sigmodontis/* mouse model cannot be offered by other models such as *B. malayi*/ mouse/ jird, *O. volvulus*/ cattle.

The core strategy to design present vaccines is to enhance Th2-biased immune responses characterized by the increased IgG1, IL4, IL5, IL13 and decreased IgG2a as well as IFNγ (see chapter 1 and 3). Although the synergism of both Th1 and Th2 responses might contribute to the successful protection, the Th2-biased immune responses have been shown

to be the pre-dominant responses donated to the reduction of worm burden by massive researches (see section 1.2). Current experiments tried to increase Th2 responses by the application of adjuvants of IL4, MIP1α and Flt3L, which had been proved to augment the immune responses individually or synergistically by Simon Babayan in our previous works. Results showed their combination assisted the enhancement of Th2 responses following current vaccinations, which is why these adjuvants were used in all experiments in the same amounts. Besides, a new exploration to test a new cytokine adjuvant IL33 had been performed (data not shown) based on the observation that IL33 recombinant protein increases the survival of eosinophils by foreign administration. However, the exploration failed. Even so, more works are worth of investigating on IL33 as cytokine adjuvant in future works.

Regardding dual vaccine comprising DECADDALT and DECCPImu, the main contributors to the protective immunity were the high level of IgG1, increased IL5 and IL13 instead of IgG2a and eosinophils. For the tri-vaccine, Tri-VAH and Tri-TPX, the protective immunity attributed to high level of IgG1, increased IL5 or IL13 (not all Th2 cytokines), eosinophils and decreased IgG2a as well as IFNγ. IgG1 is the antibody represents Th2 response in mouse immune responses and it has been described relates to the ADCC mechanism and is vital to the worm killing (discussed in chapter3), thus the high level of IgG1 in current experimental mice, with no doubts, attributed to the high protective immunity. However, one might argue the quite high level of IgG1, more than 10^5, might result in hyper-responses and increase the possibility that exacerbates host health. It cannot be denied that the IgG1 level was high on dual vaccine, but it was also high on controls, thus comparatively, the IgG1 on dual vaccine was normal. Another explanation might be the difference of coating protein. When IgG in single vaccine was measured, the relative recombinant protein was used, it only recognizes specific protein. However, the whole raw proteins secreted from *L. sigmodontis* adults were used when dual vaccine was measured considering the dual vaccine contains two different

candidates which need to recognize two different proteins. In the whole raw proteins, there were many other unknown proteins which might interact with antigens, consequently promoted the level of IgG1, similar phenomenon had been demonstrated in cocktail vaccine experiments (see chapter 5). However, the specific reasons have not been investigated yet.

Th2 cytokines have been described to be essential to worm or Mf killing (discussed in chapter 3). However, present data from single vaccine of DECADDALT and DECCPImu in chapter 3 indicated that despite IL4, IL5, and IL13 were significantly increased, the worm burden was not decreased significantly. In surprise, in dual and cocktail vaccines, despite the IL5 and/ or IL13 increased but not significantly, the worm burden was decreased significantly, suggesting that one or two factors are not fully contributed to final protection, the key might be the co-reaction among all factors. In addition, the decreased IFNγ was produced in dual and cocktail vaccine accompanied with the increased Th2 cytokines, (although it increased in single vaccine of TPX), suggesting the Th2-biased responses were successfully induced as designed.

Another phenomenon worth mentioning is the cytokine productions, which were strong in single and dual vaccine experiments, were quite week even none in cocktail vaccine experiments. The reasons are unknown so far. However, besides the experimental errors and long interval (longer than 3 months) between the second immunization and challenge, the most possible factor might be the effects of mixture of antigens on host immune system, in other words, the antigens interactions might result in the weak production of cytokines, but it has not been investigated yet.

The success of the ADDALT and CPImu vaccination is clearly associated with the removal of a sequence expressing a “regulatory” or “suppressive” domain, 46 aa domain in the case of ADDALT and a single site mutation (Asn66 to Lys66) for CPImu, which abolish any

stimulation of host modulatory responses. The combination of DECADDALT and DECCPImu increased such power of properties than single immunization (chapter 3) and resulted in the final high protection, suggesting the removed suppression of single antigen might not enough to augment the immune responses and the combined deletion of inhibitions might be the base for further vaccine development. Therefore, subsequent cocktail vaccinations based on ADDALT and CPImu candidates had been shown that this combination could assist other candidates such as VAH or TPX to elevate the immune responses and eventually achieved protections. This had been supported by present single vaccine of VAH or TPX, which could not achieve protection whereas strong Th2-biased responses were induced respectively. One might be curious that whether the Treg responses were involved in or not during the removal of immunosuppression of ALT and CPI2. It cannot be denied that the key role of Tregs during filarial infection. The severe bias of the balance among Tregs, Th1, Th2 and Th17 might lead to the disorders of immune system. Although current candidates achieved the similar effects to the effects that Tregs being moved, these candidates are not involved in the airway of Tregs, in other words, these removals of suppression just circumvented the immune responses. If wants to depress the Tregs, the way by targeting the key gene in its airway such as Eos or RUNX3 might work (see 1. 2).

Present results illustrate the fine depth to which analysis of antigens must be taken to identify epitopes that drive protection. This implies that a great deal more effort should be applied to the exploration of the immunogenicity of pathogens. Fortunately, new knowledge that can provide information on genomes and transcriptsomes of pathogens can now be used combined with new methods in bioinformatics. It should be possible to identify new and additional vaccine targets.

Present data exhibited that DECADDALT, which targets DEC205 receptor on the surface of DCs by the encoded ADDALT gene on DEC205 mAb that can specific recognize DEC205

receptor, elicited higher level of IgG1, Th2 cytokines IL4, IL5, IL13 as well as reduced worm burden with comparison to the pcDNAADDALT, which backbone carried on DEC205 mAb hereby cannot recognize DEC205 receptor, suggesting that the immune responses had been augmented by targeting to DCs via DEC205 receptor recognition. This is consistent to the report from Nchinda G and colleagues (Nchinda G *et al.*, 2010; Nchinda G *et al.*, 2008) that the DNA vaccine which encodes HIV gag p41-scFv DEC205 fusion protein generated 10-fold higher antibody and the mice were protected. In addition, subsequent findings on DECCPImu, DECVAH and DECTPX had provided more supports. For instance, DECCPImu induced remarkable higher IgG1, IL4, IL5, IL13 and increased IgE, IgG2a as well as decreased IL10, IFNγ in contrast to pcDNACPImu, despite of the lack of protections. However, the combination of DECADDALT and DECCPImu finally obtained significant reduction of worm burden. Moreover, both DECVAH and DECTPX evoked stronger immune responses than pcDNAVAH and pcDNATPX, respectively. All above results showed consistence with previous report from Demangel C *et al*.(2005) that the use of scDEC205 could improve DNA vaccination. DCs are known to express several receptors with the potential to enhance antigen uptake (Figdor CG *et al.*, 2002), the DEC205 receptor has been shown to deliver many different proteins for antigen presentation on both MHC I and II products (Boscardin SB *et al*., 2006; Soares H *et al.*, 2007). In contrast, other antigen uptake receptors are preferentially expressed on different DC subsets with distinct antigen-presenting properties. In addition, DEC205 targeting may has unique merits that the subset of DCs targeted by DEC205 in mice is specialized to process antigens on MHC I. Moreover, DEC205 targeting can mediate immunization of MHC II-restricted $CD4^+$ helper T cells (Bonifaz LC *et al.*, 2004; Boscardin SB *et al*., 2006; Trumpfheller C *et al.*, 2006). This has been also proved by Nchinda G and colleagues (Nchinda G *et al.*, 2010) that DEC-targeted protein vaccine could enhance antigen specific $CD4^+$ helper T cells. Regarding to present works, despite the change of $CD4^+$ helper cells post DNA vaccination was not measured, the profiles of Th2 cytokines during this process might provide some clues which might reflect the functions of $CD4^+$ helper cells.

The cells recruited in pleural cavity 60 days after the challenge infection have been analyzed, as discussed in Chapter 3, macrophages and neutrophils were involved in the worm killing correspond to the dual vaccination. These cells have been well-recorded to be responsible to host defense against invading pathogens (see section 3.3). Likewise, numbers of neutrophils recruited by the single vaccine of DECVAH, DECTPX and cocktail vaccine of Tri-vaccine and Tri-VAH showed similar trends to dual vaccine, but they were not significantly increased after vaccinations. However, numbers of macrophages in these experiments showed inverse trends when compared to dual vaccine. Whether this opposite phenomenon impacted the worm killing negatively has not been investigated. For now it cannot be answered. The possibility might be the different natures of antigens and the co-reactions among different antigens which may lead to inverse of bio-functions. However, whether these cells are associated with the Mf killing has not been answered clearly in present experiments. Data showed that all vaccines except the single vaccine of DECVAH and DECTPX resulted in the significant reduction of Mf in the blood in mice, but analysis in present study showed no correlation between these cells and the Mf killing. This is not consistent with studies about onchocerciasis. In untreated generalized infection with onchocerciasis (GEO), macrophages, eosinophils are correlated with the dead Mf (Büttner DW, Racz P, 1983) and the presence of Mf increased the accumulation of eosinophils in nodules (Wildenburg G *et al.*, 1996). Besides, the neutrophils have also been reported to participate the immune attack to Mf (Gutierrez-Pena EJ *et al.*, 1996). Several *in vitro* researches have also demonstrated that the eosinophils and neutrophils have the capability to mediate the Mf killing in infective individuals (Greene BM *et al.*,1981; Johnson EH *et al.*, 1994). Apart from immune cells, the reasons associated with the Mf reduction in current study were assumed to be the consequence of sub-lethal effects on reproduction of the adults (Babayan SA *et al*., 2005), but the damage of embryogenesis in female worms has not been examed in current experiments. However, experiments on onchocerciasis using bovine model indicated this effects might not be simply a consequence of decreased adult female worms, because in the vaccinated cattle the Mf numbers increase was associated with the female density (Tchakouté VL *et al.*, 2006).

This might be the result of antigenic cross-reactivity of antigens from distinct life cycle stages base on the observation that successful anti-Mf immunity against *O. lienalis* was induced by iL3 vaccination in mice (Townson S *et al*., 1984). However, all antigens used in present study mainly derive from adult or L3 stages, even there is cross-reactivity from Mf, that effects are presumed to be marginal. Therefore, the specific answers have to be made after the further investigated in future works.

In designing vaccines against filarial infection, it will be extremely important to remember that pathology associated with filarial infection is also a Th2 driven hypersensitivity (King CL *et al.,* 1993; Maizels RM *et al.,* 1995) in which IgE may play a critical role. The ADDALT plus CPImu cocktail vaccine induced production of IgE over and above controls. However, no evidence of hypersensitivity was detected. While this is said, the *L. sigmodontis* BALB/c mouse model is probably not appropriate, as even in heavy infection, pathology is not observed. To answer questions about the possibility of CPImu and ALT being associated with hypersensitivity reactions, it will be useful to test reactivity of sera collected from human presenting with or without such responses. At an experimental level, the ability of these antigens to evoke specific anaphylactic (passive) responses in rats may be considered. Taking the DNA vaccine strategy further, the biological differences of filarial nematodes and their hosts have to be considered more seriously. Attempts to circumvent the immunosuppression caused by the molecules secreted or derived from parasites during their development in hosts have been tried in present works (chapter3, by deleting the acid domain of ALT and site direct mutation of CPI2). However, the immuno-modulation in host immunity during chronic filarial infection has also been well documented. The hypothesis is that the filariae-drived molecules attribute to the regulation of host immune response against antigens which derived from filariae, resulting in the increase of parasite survival and chronic infections (Maizels RM *et al*., 2001). To inhibit the Tregs in host immunity *in vivo* might be a possible way to avoid such parasite-inducible regulation. This is based on the findings that removal of regulatory T cell activity reversed the hyporesponsiveness and

resulted in the filarial parasite clearance *in vivo* (Taylor MD, *et al.*, 2005). Present work had been designed to inhibit Tregs by suppressing the Eos gene which is associated with the Tregs activity, results showed there were no impacts on suppression of Tregs in mice (data not shown), however, it was only tested once and this regimen had not been optimized. Besides, the success suppression of Tregs leads to the increase of Th2 cells has been observed previously by other researchers (see 1.2). Thus it cannot be ignored simply. Another biological feature noteworthy is the intracellular bacteria *Wolbachia* in filarial nematodes. Abundant evidences have showed that this kind of bacterial impacts the growth, biology and immunity of filarial nematodes and has already been proved in gene level by genomic sequencing (Fenn K, *et al.*, 2004). The molecules involved in the parasite- *Wolbachia* interactions such as heme-dependent enzymes, which are essential for energy metabolism, (Rao RU, *et al*., 2002) might be potential targets to develop novel DNA vaccines. The comprehensive system biology approachs may provide a chance to define such molecules and point the biochemical and physiological dependences that can be used to control filariasis.

In formulating vaccines, it will be important to consider the commercial manufacture processes including quality control and material resource requirements. For experimental purposes, this work has used cocktails of recombinant plasmids. This allows investigation of the contribution of individual components to the induction of immune responses. However, for commercial production, a single construction [plasmid] would be preferred, because it is a key issue for validation of process that evaluating the quality of the eventual plasmid preparation in light of its purity, safety and potency (Schleef M, 1999). The more components raise more chances to disorder the quality control process. Although the proof-of principle researches of DNA vaccine have been demonstrated in various animal models, as an instance of present dual vaccine and cocktail vaccine, to date, only few commercial DNA vaccines are licensed. The barriers to commercialization include ease way of delivery, transfection efficacy, cost of production and safety (Běláková J *et al*., 2007). Although the adverse impacts

on safety by plasmid *in vivo* are quite low, the irrational concerns about societal or ethical aspects of DNA products may discourage firms to make the commercialized products. In addition, the complicate judgment processes and multiple patents of a DNA vaccine may increase the costs, consequently discourage companies to register a novel DNA vaccine. Finally, an easier delivery system, which can increase the efficacy of DNA vaccine directly in target cells, is not now available for livestock use, although such devices have been used in mice in lab (Babiuk LA *et al.*, 2000). Thus, measures must be taken to overcome such barriers. Apart from the techniques required to enhance the efficacy and delivery methods (see 1.3), intensive and extensive publicity might be essential to put forward, which makes public understanding of the nature of DNA vaccine correctly and can be accepted rationally. Also the government policy- makers might put more concerns on the managements of patents and encouragement of firms, which may reduce the costs and processes that a DNA vaccine can finally be brought into markets, consequently leads to the rebound of registration for DNA vaccine products.

6.3 Future work

The results of this study are of significance because of the successful protection achieved by vaccination with the ADDALT plus CPImu resulting in more than 80% reduction of worm burden. It is a demonstration of the feasibility of use of a DNA vaccine against filarial infection.

This present study demonstrates the great amenability of the *L. sigmodontis*-BALB/c mouse model in development of a DNA vaccine against filarial infection. However, as is the case in most helminth models of infection, difficulties and constraints arise with measurement and assessment of worm burden. The use of just 25 or 30 infective larvae is a “challenge” for delivery and statistical analysis. Recovery of adult worms and arrestment of Mf are also very difficult to measure and irrespective of adult worm number which relates to the Mf numbers vary. Despite of these limitations, protective responses have been demonstrated.

The present study is only a preliminary stage of DNA vaccine against filarial infections. Commercial use of the ADDALT and CPImu is a long way off. There are a number of aspects that require investigation including: 1) Possible toxicity of the recombinant plasmid at a local and systemic level; 2) The exact tissue distribution of vaccine, the storage life of vaccine in host cells, the expression time and strength of vaccine. 3) The research of genetic toxicity, mainly focus on the detection of whether the plasmid DNA insertion may result in the integration into the host genome, and the consequences of DNA plasmid and its expression products on the target or non-target organs. 4) In addition, it might be necessary to determine whether the DNA vaccines can cause reproductive toxicity, which raises the need to measure samples in both male and female mice.

Furthermore, the present work measured immune responses in mice that were only given one challenge, but under field conditions, there will be multiple challenges, and it will be necessary to investigate challenge that may occur under such antigenic pressure. This work will be carried out using the *O. ochengi*-bovine model. Finally, it will be important to improve and simplify the formulation of present vaccines and DNA adjuvants and indeed consider whether use of recombinant protein of CPImu and ADDALT may not afford better protection and less manifestation of pathology.

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Appendix

Appendix 1. Bacterial host strains and related plasmid vectors used in experiments

Host strain	Genotype	Related vectors
One shot Top10 (Invitrogen UK)	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG.	pDEC-ADDALT, pDEC-CPI2, pDEC-CPImu, pDEC-VAH, pDEC-TPX, pDEC-OVA, pISO-ADDALT, pISO-CPI2, pISO-CPImu, pISO-VAH, pISO-TPX, pISO-OVA
JM109 (Promega USA)	e14–(McrA–) recA1 endA1 gyrA96 thi-1 hsdR17 (rK– mK+) supE44 relA1 Δ(lac-proAB) (F´ traD36 proAB lacIqZΔM15).	pDEC-ADDALT, pDEC-CPI2, pDEC-CPImu, pDEC-VAH, pDEC-TPX, pDEC-OVA, pISO-ADDALT, pISO-CPI2, pISO-CPImu, pISO-VAH, pISO-TPX, pISO-OVA
BL21-AI (Invitrogen UK)	F- ompT hsdSB (rB-mB-) gal dcm araB::T7RNAP-tetA), BL21(DE3) (E. coli B F– dcm ompT hsdS(rB– mB–) gal λ(DE3)	pET21b-ALT,-CPI2,-VAH, -TPX; pET29c-ALT,-CPI2,-VAH, -TPX; pET24c-ALT,-CPI2,-VAH, -TPX; pET30a-ALT,-CPI2,-VAH, -TPX;
BL21 (DE3) (Novagen, UK)	(E. coli B F– dcm ompT hsdS(rB– mB–) gal λ(DE3)	pET21b-ALT,-CPI2,-VAH, -TPX; pET29c-ALT,-CPI2,-VAH, -TPX; pET24c-ALT,-CPI2,-VAH, -TPX; pET30a-ALT,-CPI2,-VAH, -TPX;
Rosotta-gami 2 (Novagen, UK)	Δ(ara–leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsLF'[lac+ lacI q pro] gor522::Tn10 trxB pRARE23 (CamR, StrR, TetR)	pET21b-ALT,-CPI2,-VAH, -TPX; pET29c-ALT,-CPI2,-VAH, -TPX; pET24c-ALT,-CPI2,-VAH, -TPX; pET30a-ALT,-CPI2,-VAH, -TPX;
Origami (DE3) (Novagen, UK)	(ara–leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpL F'[lac+ lacI q pro] (DE3) gor522::Tn10 trxB (KanR, StrR, TetR)	pET21b-ALT,-CPI2,-VAH, -TPX; pET29c-ALT,-CPI2,-VAH, -TPX; pET24c-ALT,-CPI2,-VAH, -TPX; pET30a-ALT,-CPI2,-VAH, -TPX;

Appendix 2.1 Designed primers used in experiments

Transcript	Primer sequence	Annealing temp(°C)	Product size(bp)
LsALT	F:5'-CACCATGATGTCGGTGAAGGGTGTATTATT-3' R: 5'-TTACGAAAGTAACTTTGTTTCCAAG-3'	55	444
LsADDALT	F:5'-CACCATGAACAAAGTTTTGATAATCTTTGGC-3' R:5'-TTAATCGTATGAGCATT-3'	55	306
LsCPI2	F:5'-CACCATGATGTCGGTGAAGGGTGTATTATT-3' R: 5'-TTACGAAAGTAACTTTGTTTCCAAG-3'	53	543
LsCPImu	F: 5'-GAT AAT CAA CAG TCA AAA GAT GCG TAT CAC CTT ATG CC-3' R: 5'-GG CAT AAG GTG ATA CGC ATC TTT TGA CTG TTG ATT ATC-3'	54	543
LsVAH	F: 5'-CACCATGTATATGCCACGCGGAAAA-3' R: 5'-TTATTTGATGCATAAGCCC-3'	53	504
LsTPX	F: 5'-CACCATGACACTTGCTGGAAGC-3' R: 5'-TCAATGGTGATGGTGATG-3'	55	723
msIL33	F:5'-CACCATGAGACCTAGAATGAAGTATTCCA-3 R: 5'-TTAGATTTTCGAGAGCTTAAACATA-3'	54	801
msEOS	F:5'-GATCCCTCAGTGCCAACTCCATCAAGTTCAA GAGACTTGATGGAGTTGGCACTGAGA-3' R:5'-AGCTTCTCAGTGCCAACTCCATCAAGTCTCT TGAACTTGATGGAGTTGGCACTGAGG-3'	54	57
msEOSsc	F:5'-GATCCTACGCTCGCACATACTCCGAGTTCAAG AGACGTAGTTGATCGTGGATCGGAA-3' R:5'-AGCTTTACGCTCGCACATACTCCGAGTCTCTT GAACGTAGTTGATCGTGGATCGGAG-3'	54	57
OVA	F: 5'-CACCGGCCGCACCATGGGCT-3' R: 5'-AGGGGAAACACATCTGCC-3'	55	1167
PcDNA3.1	T7 : 5'-TAATACGACTCACTATAGGG-3' BGH: 5'-TAGAAGGCACAGTCGAGG-3'	55	

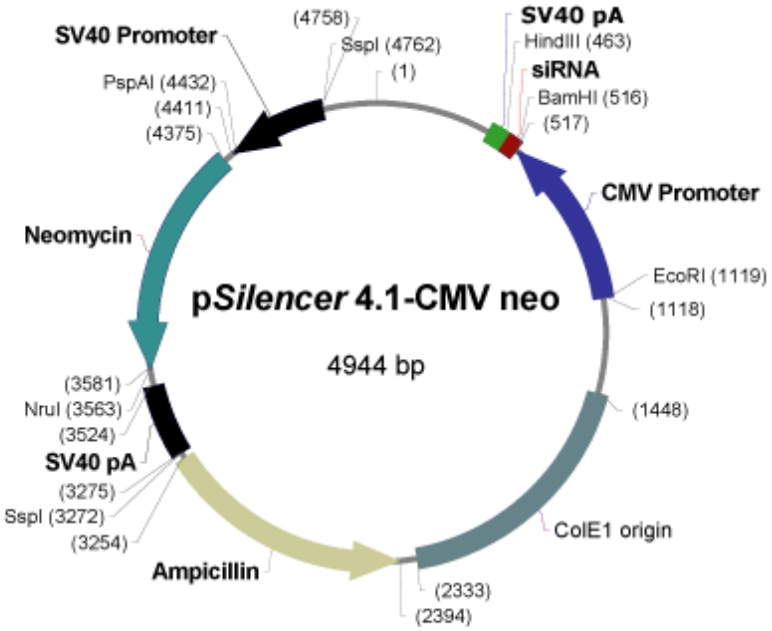
Appendix 2.2. Designed primers used in experiments (continued)

Transcript	Primer sequence	Annealing temp(°C)	Product size(bp)
LsALT expression	F:5'-GGGAATTTCATATGCAGGAAGAAGACGAGATG-3' R:5'-CGGCTCGAGATCGTATGAGCATTGCCAG-3'	55	444
LsCPI2 expression	F:5'-GGGAATTCCATATGCTGGGACACGGAAATATGG-3' R:5'-CGGCTCGAGCTGATCCGGATGGCCTTCCAAT-3'	54	543
LsVAH expression	F:5'-GGGAATTCCATATGTATATGCCACGCGGAAAA-3' R: 5'-CGGCTCGAGTTTGATGCATAAGCCC-3'	53	504
LsTPX expression	F:5'-GGGAATTCCATATGACACTTGCTGGAAGC-3 R: 5'-CGGCTCGAGATGGTGATGGTGATG-3'	55	723
pET vectors	T7 : 5'-TAATACGACTCACTATAGGG-3' T7 terminator: 5'-GCTAGTTATTGCTCAGCGG-3'	55	
DECADDALT	F:5'-AAGGAAAAAAGCGGCCGCCACCATGAACAAAGTTTTGATAATCTTTGGC-3' R: 5'-CTAGTCTAGA TTAATCGTATGAGCATT-3'	55	306
DECCPI2	F:5'-AAGGAAAAAAGCGGCCGCCACCATGATGTCGGTGAAGGGTGTATTATT-3' R:5'-CTAGTCTAGATTACGAAAGTAACTTTGTTTCCAAG-3'	53	543
DECCPImu	F:5'-AAGGAAAAAAGCGGCCGCACCATGATGTCGGTGAAGGGTGTATTATT-3' R:5'-CTAGTCTAGATTACGAAAGTAACTTTGTTTCCAAG-3'	53	543
DECVAH	F:5'-AAGGAAAAAAGCGGCCGCCACCATGTATATGCCACGCGGAAAA-3' R:5'-CTAGTCTAGATTATTTGATGCATAAGCCC-3'	54	504
DECTPX	F:5'-AAGGAAAAAAGCGGCCGCCACCATGACACTTGCTGGAAGC-3' R:5'-CTAGTCTAGATCAATGGTGATGGTGATG-3'	55	723

Appendix 3. Plasmid constructions used in experiments. Double-digested insert fragment was ligated into a backbone plasmid which was double-digested with the same enzymes, and the ligation reaction was performed with T4 DNA ligase at 16°C overnight. CACC sequence is a linker in backbone plasmid of pcDNA3.1 through which the inserted fragment carried it can be ligated into the vector.

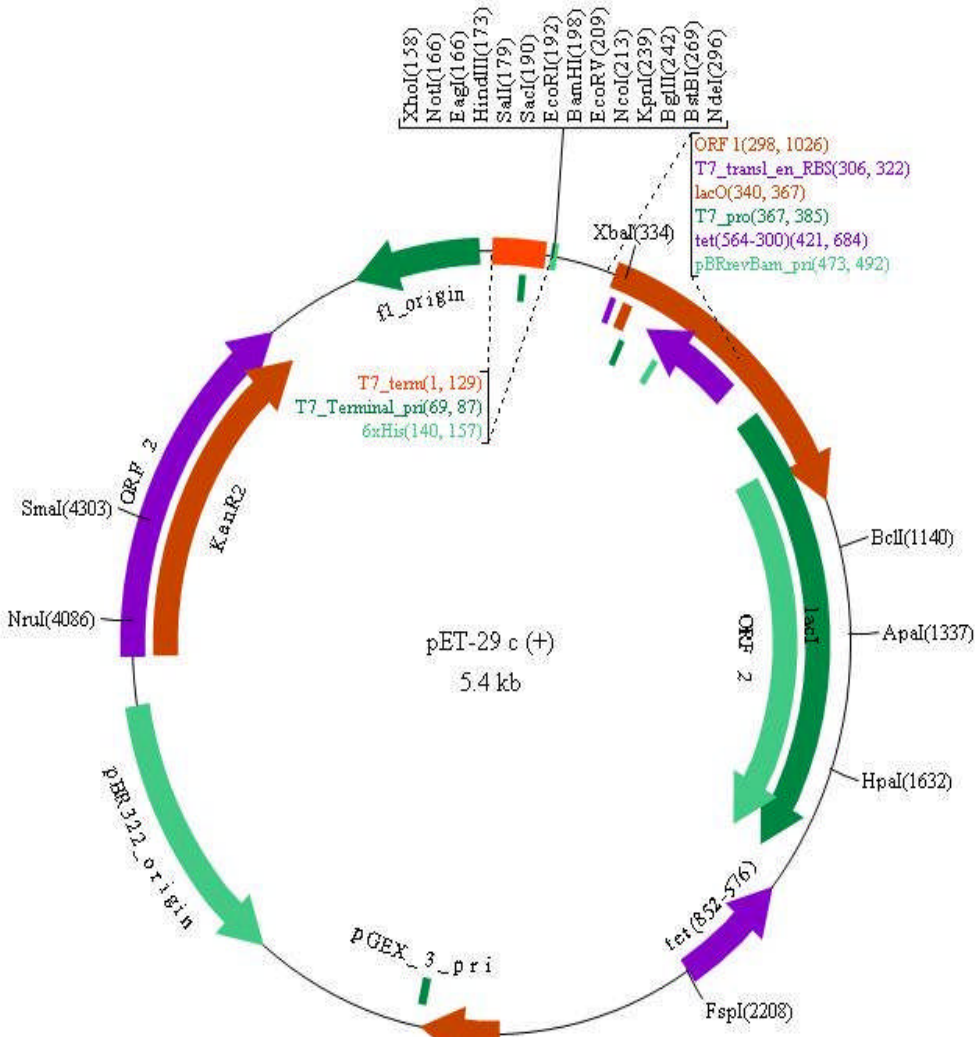
Constructed plasmid	**Backbone plasmid**	**Inserted fragment**	**Restriction enzyme**
pcDNA3.1-ALT pcDNA3.1-ADDALT pcDNA3.1-CPI2 pcDNA3.1-CPImu pcDNA3.1-VAH pcDNA3.1-TPX	pcDNA3.1	ALT (with CACC) ADDALT(with CACC) CPI2 (with CACC) CPImu (with CACC) VAH (with CACC) TPX (with CACC)	
pDEC-ADDALT pDEC-CPI2 pDEC-CPImu, pDEC-VAH pDEC-TPX	pDEC-OVA (digested with NotI and XbaI to delete OVA)	ADDALT, CPI2, CPImu, VAH, TPX	NotI and XbaI
pISO-ADDALT pISO-CPI2 pISO-CPImu pISO-VAH pISO-TPX	pISO-OVA (digested with NotI and XbaI to delete OVA)	ADDALT, CPI2, CPImu, VAH, TPX	NotI and XbaI
pET21b-ALT pET21b-CPI2 pET21b-VAH pET21b-TPX	pET21b (digested with NdeI and Xho I)	ALT, CPI2, VAH, TPX	Nde I and Xho I
pET29c-ALT pET29c-CPI2 pET29c-VAH pET29c-TPX	pET29c (digested with NdeI and Xho I)	ALT, CPI2, VAH, TPX	Nde I and Xho I
pET24a–ALT pET24a–CPI2 pET24a–VAH pET24a–TPX	pET24a (digested with NdeI and Xho I)	ALT, CPI2, VAH, TPX	Nde I and Xho I
pET30a-ALT pET30a-CPI2 pET30a-VAH pET30a-TPX	pET30a (digested with NdeI and Xho I)	ALT, CPI2, VAH, TPX	Nde I and Xho I

Appendix 4. Schematic of pSilencer 4.1-CMV neo vector. msEOS and msEOSsc (see appendix 1) were inserted into pSilencer 4.1-CMV neo vector between Hind III and BamHI to construct pSiEOS and pSiEOSSC plasmids.



CMV Promoter: 517-1118
SV40 pA signal: 400-462
SV40 Promoter: 4411-4758
Neomycin: 3581-4375
SV40 pA signal: 3275-3524
Ampicillin: 2394-3254
ColE1 origin: 1448-2333

Appendix 5. Schematic of pET29c vector. In the multi-cloning site (MCS), XhoI and NotI were chosen as enzymes and the fragment double digested by both XhoI and NotI was insected in MCS. T7 tag is at the N terminus and His tag is at the C terminus. pET21b, pET24a and pET30a have simlar structure in the MCS region and N/ C terminus to pET29c.



Appendix 6. Output of the total variance of the principal component assay.

Comp-onent	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	6.306	20.342	20.342	6.306	20.342	20.342	4.930	15.904	15.904
2	4.343	14.010	34.351	4.343	14.010	34.351	4.239	13.675	29.579
3	3.703	11.947	46.298	3.703	11.947	46.298	3.671	11.843	41.422
4	3.163	10.204	56.502	3.163	10.204	56.502	3.156	10.182	51.604

Appendix 7. Output of the rotated component matrix of the principal component

	PC1	PC2	PC3	PC4
IgE	0.064	0.011	-0.162	-0.067
anti.worm.IgG1	0.029	0.305	-0.063	-0.076
anti.ALT.IgG1	0.177	-0.060	-0.362	0.171
anti.CPI.IgG1	0.173	-0.069	-0.366	0.187
anti.worm.IgG2a	0.050	0.019	0.038	-0.012
anti.ALT.IgG2a	0.171	0.149	-0.360	-0.144
anti.CPI.IgG2a	0.162	0.153	-0.330	-0.162
IL4.PL	-0.031	-0.087	-0.119	-0.213
IL5.PL	0.055	0.126	0.255	-0.243
IL13.PL	0.061	0.170	0.143	-0.326
IFNgPL	-0.066	-0.052	0.056	-0.241
IL4.M.LN	0.213	-0.151	0.040	0.319
IL4.LsAg.LN	0.200	-0.043	0.167	0.029
IL4.aCD3.LN	0.117	0.051	0.177	0.238
IL5.M.LN	0.306	-0.093	0.157	0.116
IL5.LsAg.LN	0.308	0.079	-0.012	-0.165
IL5.aCD3.LN	0.313	-0.025	0.105	-0.092
IL13.M.LN	0.231	-0.020	0.120	0.059
IL13.LsAg.LN	0.330	0.036	0.139	-0.189
IL13.aCD3.LN	0.321	-0.029	0.013	-0.102
IFNg.M.LN	0.230	-0.078	0.211	0.207
IFNg.LsAg.LN	0.074	0.011	0.199	-0.170
IFNg.aCD3.LN	0.157	-0.132	-0.164	0.244
EosNx	0.086	0.425	-0.032	0.083
NeuNx	-0.041	0.399	0.049	0.162
MacNx	0.085	0.422	-0.108	0.068
LyMoNx	-0.113	0.118	0.252	0.347
PleCNx	0.018	0.428	0.059	0.211
Prol.M	0.232	-0.057	-0.003	0.053
Prol.LsAg	0.184	-0.002	0.158	-0.108
Prol.aCD3	0.114	-0.103	-0.109	-0.059